



US009206408B2

(12) **United States Patent**  
**Callewaert et al.**

(10) **Patent No.:** **US 9,206,408 B2**  
(45) **Date of Patent:** **Dec. 8, 2015**

(54) **MICROORGANISMS GENETICALLY  
ENGINEERED TO HAVE MODIFIED  
N-GLYCOSYLATION ACTIVITY**

(75) Inventors: **Nico Luc Marc Callewaert**,  
Hansbeke-Nevele (BE); **Wouter**  
**Vervecken**, Landskouter (BE); **Karen**  
**Jacqueline Marcel De Pourcq**, Ghent  
(BE); **Steven Christian Jozef Geysens**,  
Wannegem-Lede (BE); **Mouna Guerfal**,  
Veltem-Beisem (BE)

(73) Assignees: **Oxyrane UK Limited**, Manchester  
(GB); **VIB vzw**, Ghent (BE);  
**Universiteit Gent**, Ghent (BE)

(\*) Notice: Subject to any disclaimer, the term of this  
patent is extended or adjusted under 35  
U.S.C. 154(b) by 182 days.

(21) Appl. No.: **13/620,306**

(22) Filed: **Sep. 14, 2012**

(65) **Prior Publication Data**

US 2013/0190253 A1 Jul. 25, 2013

#### **Related U.S. Application Data**

(63) Continuation of application No. 13/095,532, filed on  
Apr. 27, 2011, which is a continuation of application  
No. 12/062,469, filed on Apr. 3, 2008, now Pat. No.  
8,026,083.

(60) Provisional application No. 60/940,212, filed on May  
25, 2007, provisional application No. 60/909,904,  
filed on Apr. 3, 2007.

(51) **Int. Cl.**

**A61K 38/16** (2006.01)  
**C12N 9/24** (2006.01)  
**C12N 9/10** (2006.01)  
**C12P 21/00** (2006.01)  
**C07K 14/00** (2006.01)  
**C12N 9/40** (2006.01)  
**C12P 21/06** (2006.01)  
**C07K 14/39** (2006.01)  
**A61K 38/00** (2006.01)

(52) **U.S. Cl.**

CPC ..... **C12N 9/2488** (2013.01); **C07K 14/00**  
(2013.01); **C12N 9/1051** (2013.01); **C12N 9/24**  
(2013.01); **C12N 9/2402** (2013.01); **C12N**  
**9/2465** (2013.01); **C12P 21/005** (2013.01);  
**C12P 21/06** (2013.01); **C12Y 204/01232**  
(2013.01); **C12Y 302/01024** (2013.01); **C12Y**  
**302/01045** (2013.01); **C12Y 302/01046**  
(2013.01); **C12Y 302/01084** (2013.01); **C12Y**  
**302/01113** (2013.01); **A61K 38/00** (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

(56) **References Cited**

#### **U.S. PATENT DOCUMENTS**

4,307,717	A	12/1981	Hymes et al.
4,352,883	A	10/1982	Lim
4,353,888	A	10/1982	Sefton
4,407,957	A	10/1983	Lim
4,704,362	A	11/1987	Itakura et al.
4,837,148	A	6/1989	Cregg
4,879,231	A	11/1989	Stroman et al.
4,882,279	A	11/1989	Cregg
4,883,666	A	11/1989	Sabel et al.
4,929,555	A	5/1990	Cregg et al.
4,968,733	A	11/1990	Muller et al.
4,976,859	A	12/1990	Wechs
5,084,350	A	1/1992	Chang et al.
5,158,881	A	10/1992	Aebischer et al.
5,272,070	A	12/1993	Lehrman et al.
5,284,761	A	2/1994	Aebischer et al.
5,585,089	A	12/1996	Queen et al.
5,798,113	A	8/1998	Dionne et al.
5,800,828	A	9/1998	Dionne et al.
6,110,703	A	8/2000	Egel et al.
6,265,185	B1	7/2001	Muller et al.
6,699,658	B1	3/2004	Wittrup et al.
6,803,225	B2	10/2004	Contreras et al.
6,872,392	B2	3/2005	Nakamura et al.
7,029,872	B2	4/2006	Gerngross
7,262,287	B2	8/2007	Kang et al.
7,326,681	B2	2/2008	Gerngross
7,390,884	B2	6/2008	Segal et al.
7,422,742	B2	9/2008	Greenfeder et al.
7,422,890	B2	9/2008	Gopalakrishnakone et al.
7,431,927	B2	10/2008	Couto et al.
7,442,772	B2	10/2008	Goddard et al.
7,449,308	B2	11/2008	Gerngross et al.
7,785,856	B2	8/2010	LeBowitz et al.
8,026,083	B2	9/2011	Callewaert et al.

(Continued)

#### **FOREIGN PATENT DOCUMENTS**

AU	2012206984	8/2012
EP	1408117	4/2004

(Continued)

#### **OTHER PUBLICATIONS**

Vega et al, Journal of Basic Microbiology; vol. 28, 1988, pp. 371-379.\*

(Continued)

*Primary Examiner* — Joanne Hama

*Assistant Examiner* — Fozia Hamud

(74) *Attorney, Agent, or Firm* — Fish & Richardson P.C.

(57) **ABSTRACT**

Described herein are methods and genetically engineered cells useful for producing an altered N-glycosylation form of a target molecule. Also described are methods and molecules with altered N-glycosylation useful for treating a variety of disorders such as metabolic disorders.

(56)

## References Cited

## U.S. PATENT DOCUMENTS

8,597,906	B2	12/2013	Callewaert et al.
2002/0137125	A1	9/2002	Zhu
2003/0147868	A1	8/2003	Treco et al.
2003/0186374	A1	10/2003	Huften et al.
2004/0018588	A1	1/2004	Contreras et al.
2004/0018590	A1	1/2004	Gerngross et al.
2005/0014270	A1*	1/2005	Picattaggio et al. .... 435/483
2005/0064539	A1	3/2005	Chiba et al.
2005/0170452	A1	8/2005	Wildt et al.
2005/0265988	A1	12/2005	Choi et al.
2006/0014264	A1	1/2006	Sauer et al.
2006/0030521	A1	2/2006	Defrees et al.
2006/0040353	A1	2/2006	Davidson et al.
2006/0148039	A1	7/2006	Kobayashi et al.
2006/0286637	A1	12/2006	Hamilton
2007/0037248	A1	2/2007	Bobrowicz et al.
2008/0171359	A1	7/2008	Botes et al.
2009/0069232	A1	3/2009	Callewaert et al.
2009/0186011	A1	7/2009	Vellard et al.
2010/0291059	A1	11/2010	Sakuraba et al.
2011/0201540	A1	8/2011	Callewaert et al.
2011/0207676	A1	8/2011	Callewaert et al.
2012/0135461	A1	5/2012	Cook et al.
2013/0053550	A1	2/2013	Geysens et al.
2013/0096281	A1	4/2013	Ryckaert et al.
2013/0158239	A1	6/2013	Callewaert et al.
2013/0195835	A1	8/2013	Callewaert et al.
2013/0243746	A1	9/2013	Vervecken et al.
2013/0267473	A1	10/2013	Piens et al.
2013/0295603	A1	11/2013	Piens et al.
2015/0031081	A1	1/2015	Vervecken et al.

## FOREIGN PATENT DOCUMENTS

FR	2954349	6/2011
JP	57-054588	4/1982
JP	2004-313074	11/2004
KR	20040062304	7/2004
WO	WO 92/19195	11/1992
WO	WO 95/05452	2/1995
WO	WO 96/04378	2/1996
WO	WO 96/21038	7/1996
WO	WO 98/01473	1/1998
WO	WO 98/01535	1/1998
WO	WO 98/48025	10/1998
WO	WO 99/36569	7/1999
WO	WO 99/37758	7/1999
WO	WO 01/49830	7/2001
WO	WO 02/18570	3/2002
WO	WO 03/029456	4/2003
WO	WO 03/056914	7/2003
WO	WO 2004/003194	1/2004
WO	WO 2004/074458	9/2004
WO	WO 2004/074461	9/2004
WO	WO 2004/074498	9/2004
WO	WO 2004/074499	9/2004
WO	WO 2005/100584	10/2005
WO	WO 2005/106010	11/2005
WO	WO 2008/100816	8/2008
WO	WO 2008/120107	10/2008
WO	WO 2009/033507	3/2009
WO	WO 2010/099195	9/2010
WO	WO 2011/039634	4/2011
WO	WO 2011/061629	5/2011
WO	WO 2012/042386	4/2012
WO	WO 2012/042387	4/2012
WO	WO 2013/098651	7/2013

## OTHER PUBLICATIONS

Australian Patent Office, Search Report and Written Opinion, Singapore Application No. 200906604-4, mailed Sep. 22, 2010, 19 pages.

De Pourcq et al., "Engineering *Yarrowia lipolytica* to produce glycoproteins homogeneously modified with the universal

Man3GlcNAc2 N-glycan core," *PLoS One*, 7(6):e39976, 12 pages, Epub Jun. 29, 2012.

Park et al., "Essential role of YIMPO1, a novel *Yarrowia lipolytica* homologue of *Saccharomyces cerevisiae* MNN4, in mannosylphosphorylation of N- and O-linked glycans," *Appl Environ Microbiol.*, 77(4):1187-1195, Epub Dec. 23, 2010.

Extended European Search Report for Application No. 12000268.8 dated Jul. 12, 2013, 13 pages.

"*Arxula adeninivorans*," Wikipedia [online] Jan. 13, 2010 [retrieved on Jan. 31, 2010]. Retrieved from the Internet: <URL: [http://en.wikipedia.org/wiki/Arxula\\_adeninivorans](http://en.wikipedia.org/wiki/Arxula_adeninivorans)>, 2 pages.

"Eukaryotes Genomes—*Yarrowia Lipolytica*," The European Bioinformatics Institute [online] [retrieved on Jun. 26, 2012]. Retrieved from the Internet: <URL: [http://www.ebi.ac.uk/2can/genomes/eukaryotes/Yarrowia\\_lipolytica.html](http://www.ebi.ac.uk/2can/genomes/eukaryotes/Yarrowia_lipolytica.html)>, 1 page.

Aebi et al., "Cloning and characterization of the ALG3 gene of *Saccharomyces cerevisiae*," *Glycobiology*, vol. 6, No. 4, pp. 439-444, (1996).

Akeboshi et al., "Production of Recombinant Beta-Hexosaminidase A, a Potential Enzyme for Replacement Therapy for Tay-Sachs and Sandhoff Diseases, in the Methylophilic Yeast *Ogataea minuta*", *Appl. Environ. Microbiol.*, 73(15):4805-4812 (2007).

Alessandrini et al., "Alterations of Glucosylceramide-b-Glucosidase Levels in the Skin of Patients with Psoriasis Vulgaris," *J. Invest. Dermatol.*, 23(6):1030-1036, (2004).

Authorized Officer Cecile Chatel, International Preliminary Report on Patentability in PCT/IB2008/001814 mailed Oct. 15, 2009, 16 pages.

Bagiyan et al., "The Action of  $\alpha$ -Mannosidase from *Oerskovia* sp. on the Mannose-Rich O-Linked Sugar Chains of Glycoproteins," *Eur. J. Biochem.*, 249(1):286-292, (1997).

Baharaeen and Vishniac, "A fixation method for visualization of yeast ultrastructure in the electron microscope," *Mycopathologia*, 77(1):19-22, (1982).

Ballou, "Isolation, characterization, and properties of *Saccharomyces cerevisiae* mnn mutants with nonconditional protein glycosylation defects," *Methods in Enzymology*, vol. 185, pp. 440-470, (1990).

Barnay-Verdier et al., "Identification and characterization of two  $\alpha$ -1,6-mannosyltransferases, Anl1p and Och1p, in the yeast *Yarrowia lipolytica*", *Microbiology*, 150:2185-2195 (2004).

Barth and Gaillardin, "Physiology and genetics of the dimorphic fungus *Yarrowia lipolytica*," *FEMS Microbiology Reviews*, 19(4):219-237, Apr. 1997 [print], Jan. 2006 [online].

Bennetzen and Hall, "Codon Selection in Yeast," *J. Biol. Chem.*, 257(6):3026-3031, (1982).

Bentley et al., "Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2)," *Nature*, 417:141-147, (May 2002).

Bobrowicz et al., "Engineering of an artificial glycosylation pathway blocked in core oligosaccharide assembly in the yeast *Pichia pastoris*: production of complex humanized glycoproteins with terminal galactose", *Glycobiology*, 14(9):757-766 (2004).

Boisrame et al. "Sls1p, an endoplasmic reticulum component, is involved in the protein translocation process in the yeast *Yarrowia lipolytica*," *J. Biol. Chem.* 271(20):11668-75, (1996).

Brethauer, "Genetic engineering of *Pichia pastoris* to humanize N-glycosylation of proteins," *TRENDS in Biotechnology*, 21(11): 459-462, (Nov. 2003).

Burda et al., "Ordered assembly of the asymmetrically branched lipid-linked oligosaccharide in the endoplasmic reticulum is ensured by the substrate specificity of the individual glycosyltransferases", *Glycobiology*, 9(6):617-625 (1999).

Burton and Harding, "Hydrophobic charge induction chromatography: salt independent protein adsorption and facile elution with aqueous buffers," *J. Chromatogr. A* 814(1-2):71-81, (Jul. 1998).

Callewaert et al., "Use of HDEL-tagged *Trichoderma reesei* mannosyl oligosaccharide 1,2- -D-mannosidase for N-glycan engineering in *Pichia pastoris*," *FEBS Letters*, 503:173-178 (2001).

Callewaert et al., "Ultrasensitive profiling and sequencing of N-linked oligosaccharides using standard DNA-sequencing equipment," *Glycobiology* 11(4):275-281, (Apr. 2001).

(56)

## References Cited

## OTHER PUBLICATIONS

Caroline, Berger, European Patent Office, Examination Report, EP Application No. 08-776-351.2, dated Jan. 22, 2010, 4 pages.

Carter et al., "Humanization of an anti-p185HER2 antibody for human cancer therapy," *Proc Natl Acad Sci USA*, 89(10): 4285-4289, (May 1992).

Chiba et al., "Production in yeast of alpha-galactosidase A, a lysosomal enzyme applicable to enzyme replacement therapy for Fabry disease", *Glycobiology*, 12(12):821-828 (2002).

Choi et al. "Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast *Pichia pastoris*," *Proc. Natl. Acad. Sci. USA*, 100(9):5022-5027, (Apr. 2003).

Cipollo and Trimble, "The accumulation of Man(6)GlcNAc(2)-PP-dolichol in the *Saccharomyces cerevisiae* Deltaalg9 mutant reveals a regulatory role for the Alg3p alpha1,3-Man middle-arm addition in downstream oligosaccharide-lipid and glycoprotein glycan processing," *J Biol Chem*.275(6):4267-4277, (Feb. 2000).

Cobucci-Ponzano et al., "The molecular characterization of a novel GH38 alpha-mannosidase from the crenarchaeon *Sulfolobus solfataricus* revealed its ability in de-mannosylating glycoproteins," *Biochimie.*, 92(12):1895-1907, (Aug. 2010).

Codon usage table: *Yarrowia lipolytica* CLIB122 [gbpln]: 5967 CDS's (2945919 codons), *Codon Usage Database* [online], [retrieved on Jul. 10, 2012]. Retrieved from the Internet:< URL: <http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=284591>>, 1 page.

Connock et al., "A systematic review of the clinical effectiveness and cost-effectiveness of enzyme replacement therapies for Fabry's disease and mucopolysaccharidosis type 1," *Health Technol Assess.*, 10(20):iii-iv, ix-113, (2006).

Database UniProt[Online] Aug. 1, 1998, "SubName: Full= Putative secreted protein;" XP002628929 retrieved from EBI accession No. UNIPROT:069822 Database accession No. 069822, 3 pages.

Database UniProt[Online] Jul. 11, 2006, "SubName: Full= Alpha-1, 2-mannosidase, putative; Flags: Precursor;" XP002628931 retrieved from EBI accession No. UNIPROT:Q1ASW5 Database accession No. Q1ASW5, 2 pages.

Database UniProt[Online] Apr. 29, 2008, "SubName: Full= Putative uncharacterized protein;" XP002628930 retrieved from EBI accession No. UNIPROT:B1BZG6 Database accession No. B1BZG6, 2 pages.

Database Accession No. P41546, UniProt (online), "RecName: Full Transcriptional Activator HAC1"; XP002509286.

Davies et al, "Nomenclature for sugar-binding subsites in glycosyl hydrolases," *Biochem. J.*, 321:557-559 (1997).

Eric Toh Hock Chye, Assistant Registrar for Registrar of Patents Singapore, Singapore Search Report & Written Opinion, dated Oct. 26, 2010.

Fickers et al. "New disruption cassettes for rapid gene disruption and marker rescue in the yeast *Yarrowia lipolytica*," *J. Microbiol. Methods*. 55(3):727-737, (Dec. 2003).

Fickers et al., "Carbon and nitrogen sources modulate lipase production in the yeast *Yarrowia lipolytica*," *J. Of Applied Microbiology*, vol. 96, No. 4, pp. 742-749, (2004).

Fickers, P. et al. "Hydrophobic substrate utilization by the yeast *Yarrowia lipolytica* and its potential applications," *FEMS Yeast Research*, Apr. 2005, vol. 5, No. 6-7, pp. 527-543.

Freire et al. "Efficient monitoring of enzymatic conjugation reaction by surface-enhanced laser desorption/ionization time of flight mass spectrometry for process optimization," *Bioconjug. Chem.* 17(2):559-564, (2006).

Fujita and Takegawa, "Chemoenzymatic Synthesis of Neoglycoproteins Using Transglycosylation with Endo-Beta-N-acetylglucosaminidase A," *Biochem. Biophys. Res. Commun.*, 282(3):678-682, (Apr. 2001).

Gao et al. "UpGene: Application of a web-based DNA codon optimization algorithm," *Biotechnol. Prog.*, 20(2): 443-448, (2004).

Gellissen, et al., New yeast expression platforms based on methylotrophic *Hansenula polymorpha* and *Pichia pastoris* and on

dimorphic *Arxula adenivorans* and *Yarrowia lipolytica*—A comparison, *FEMS Yeast Research*, Aug. 24, 2005, pp. 1079-1096.

Genbank Accession No. XM\_502922 GI:50550898, "Yarrowia lipolytica YALI0D17028p (YALI0D17028g) mRNA, complete cds," Oct. 29, 2008, 2 pages.

Genbank Accession No. XM\_503217 GI:50551486, "Yarrowia lipolytica YALI0D24101p (YALI0D24101g) mRNA, complete cds," Oct. 29, 2008, 2 pages.

Genbank Accession No. AAF34579 GI:6979644, "1,2-a-D-mannosidase [Trichoderma reesei]" Feb. 16, 2000, 1 page.

Genbank Accession No. AAO78636.1 GI:29340846, putative alpha-1,2-mannosidase [Bacteroides thetaiotaomicron VPI-5482] Feb. 8, 2011, 2 pages.

Genbank Accession No. AAO79070.1 GI:29341282, "putative alpha-1,2-mannosidase [Bacteroides thetaiotaomicron VPI-5482]," Feb. 8, 2011, 2 pages.

Genbank Accession No. AAO79099.1, "putative alpha-1,2-mannosidase.[Bacteroides thetaiotaomicron VPI-5482]," Feb. 8, 2011, 2 pages.

Genbank Accession No. AF212153 GI:6979643, "Hypocrea jecorina 1,2-a-D-mannosidase (MDS1) mRNA, complete cds," Feb. 16, 2000, 2 pages.

Genbank Accession No. AF441127 GI:16974782, "Yarrowia lipolytica Mnn9p (mnn9) gene, complete cds," Apr. 11, 2003, 2 pages.

GenBank Accession No. AJ563920 GI:38488499, "Yarrowia lipolytica och1 gene for alpha 1,6 mannosyltransferase," Nov. 20, 2003, 2 pages.

GenBank Accession No. AJ865333 GI:56266607, "Trypanosoma brucei brucei glcasella gene for glucosidase II alpha subunit precursor," Oct. 25, 2005, 2 pages.

GenBank Accession No. BAA08634 GI:1171477, "alpha-mannosidase [Aspergillus saitoi]" Feb. 10, 1999, 1 page.

GenBank Accession No. NP\_630514 GI:21224735, "hypothetical protein SCO6428 [Streptomyces coelicolor A3(2)]," Jan. 19, 2012, 3 pages.

GenBank Accession No. NP\_812442 GI:29348939, "alpha-1,2-mannosidase [Bacteroides thetaiotaomicron VPI-5482]" Jan. 20, 2012, 2 pages.

Genbank Accession No. XM\_499811 GI:50543289, "Yarrowia lipolytica YALI0A06589p (YALI0A06589g) mRNA, complete cds," Oct. 29, 2008, 2 pages.

GenBank Accession No. XM\_500574 GI:50546093, "Yarrowia lipolytica YALI0B06600p (YALI0B06600g) mRNA, complete cds," Oct. 29, 2008, 2 pages.

Genbank Accession No. XM\_500811 GI:50546682, "Yarrowia lipolytica YALI0B12716p (YALI0B12716g) mRNA, complete cds," Oct. 29, 2008, 2 pages.

GenBank Accession No. XM\_503488 GI:50552026, "Yarrowia lipolytica YALI0E03190p (YALI0E03190g) mRNA, complete cds," Oct. 29, 2008, 2 pages.

GenBank Accession No. YP\_003013376 YP\_003013376, "alpha-1,2-mannosidase [*Paenibacillus* sp. JDR-2]" Jun. 15, 2012, 3 pages.

GenBank Accession No. YP\_003120664 GI:256420011, "alpha-1,2-mannosidase [Chitinophaga pinensis DSM 2588]," Jun. 18, 2012, 2 pages.

GenBank Accession No. YP\_003584502 GI:295133826, "alpha-1,2-mannosidase [Zunongwangia profunda SM-A87]," Nov. 21, 2011, 2 pages.

GenBank Accession No. Z49631 GI:1015863, "S.cerevisiae chromosome X reading frame ORF YJR131w," Aug. 11, 1997, 2 pages.

GenBank Accession No. ZP\_01061975 GI:86143590, "putative alpha-1,2-mannosidas [Leeuwenhoekiella blandensis MED217]," Nov. 9, 2010, 1 page.

GenBank Accession No. ZP\_01885202 GI:149279069, "putative alpha-1,2-mannosidase [*Pedobacter* sp. BAL39]," Nov. 9, 2010, 1 page.

GenBank Accession No. ZP\_02866543 GI:169349605, "hypothetical protein CLOSP1\_00343 [Clostridium spiroforme DSM 1552]," Nov. 9, 2010, 2 pages.

GenBank Accession No. ZP\_03677957 GI:224537418, "hypothetical protein BACCELL\_02296 [Bacteroides cellulosilyticus DSM 14838]," Nov. 10, 2010, 1 page.

(56)

## References Cited

## OTHER PUBLICATIONS

- GenBank Accession No. ZP\_04848482 GI:253571075, "conserved hypothetical protein [*Bacteroides* sp. 1\_1\_6]" Jun. 9, 2010, 2 pages.
- GenBank Accession No. ZP\_05522540 GI:256784109, "secreted protein [*Streptomyces lividans* TK24]," Dec. 9, 2010, 2 pages.
- GenBank Accession No. ZP\_06527366 GI:289767988, "secreted protein [*Streptomyces lividans* TK24]" Oct. 26, 2010, 3 pages.
- GenBank Accession No. ZP\_07083984 GI:300774115, "probable alpha-1,2-mannosidase [*Sphingobacterium spiritivorum* ATCC 33861]," Dec. 1, 2010, 1 page.
- Gentzsch and Tanner, "The PMT gene family: protein O-glycosylation in *Saccharomyces cerevisiae* is vital," *Embo J*, 15(21):5752-5759, (1996).
- Gerngross, "Advances in the production of human therapeutic proteins in yeasts and filamentous fungi," *Nature Biotech.*, 22(11):1409-1414, (2004).
- Ghaemmaghami et al., "Global analysis of protein expression in yeast," *Nature*, vol. 425, No. 6959, pp. 737-741, (Oct. 2003).
- Gonzalez and Jordan, "The alpha-mannosidases: Phylogeny and adaptive diversification," *Mol Biol Evol.*, 17(2):292-300, (Feb. 2000).
- Gossen and Bujard, "Studying gene function in eukaryotes by conditional gene inactivation," *Ann. Rev. Genetics* 36:153-173, (2002).
- Grinna and Robbins, "Substrate specificities of rat liver microsomal glucosidases which process glycoproteins," *J. Biol. Chem.*, 255(6):2255-2258, (1980).
- Guarente et al., "A GAL10-CYC1 hybrid yeast promoter identifies the GAL4 regulatory region as an upstream site," *Proc. Natl. Acad. Sci. USA* 79(23):7410-7414, (1982).
- Hamilton and Gerngross, "Glycosylation engineering in yeast: the advent of fully humanized yeast," *Curr Opin Biotechnol.*, 18(5):387-392, (Oct. 2007).
- Henderson and Finn, "Human tumor antigens are ready to fly," *Adv Immunol.*, 62:217-256, (1996).
- Hermans et al., "Human lysosomal alpha-glucosidase: functional characterization of the glycosylation sites," *Biochem J.*, 289 ( Pt 3):681-686, (Feb. 1993).
- Hinnen et al. "Transformation of yeast," *Proc. Nat. Acad. Sci. USA* 5(4):1929-1933, 1978.
- Howard et al., "Identification of the Active Site Nucleophile in Jack Bean alpha-Mannosidase Using 5-Fluoro-beta-L-Gulosyl Fluoride," *J. Biol. Chem.*, 273(4):2067-2072, (1998).
- Hudson and Kortt, "High avidity scFv multimers; diabodies and triabodies," *J. Immunol. Methods*, 231(1-2):177-189, (1999).
- Hutson et al. "Engineered antibodies take center stage," *Hum. Antibodies*, 10(3-4):127-142, (2001).
- Ichishima et al., "Molecular and enzymic properties of recombinant 1,2-alpha-mannosidase from *Aspergillus saitoi* overexpressed in *Aspergillus oryzae* cells," *Biochem. J.*, 339: 589-597, (1999).
- Ito et al., "Transformation of intact yeast cells treated with alkali cations," *J. Bacteriol.*, 153(1):163-168, (1983).
- Kornfeld and Kornfeld, "Assembly of asparagine-linked oligosaccharides," *Annu Rev Biochem.*, 54:631-664, (1985).
- Kotula and Curtis, "Evaluation of foreign gene codon optimization in yeast: expression of a mouse Ig kappa chain," *Biotechnology (NY)*, 9(12):1386-1389, (1991).
- Kuroda et al., "Production of Man5GlcNAc2-type sugar chain by the methylotrophic yeast *Ogataea minuta*," *FEMS Yeast Res.* 6:1052-1062 (2006).
- Laroy et al., "Glycome mapping on DNA sequencing equipment," *Nature Protocols*, 1: 397-405 (2006).
- Lee and Park, "Enzymatic in vitro glycosylation using peptide-N-glycosidase F," *Enzyme and Microbial Technology* 30(6):716-720, (2002).
- Liao et al., "Cloning, expression, purification, and characterization of the human broad specificity lysosomal acid alpha-mannosidase," *J. Biol Chem.*, 271(45):28348-28358, (Nov. 1996).
- Lobsanov et al., "Modulation of activity by Arg407: structure of a fungal alpha-1,2-mannosidase in complex with a substrate analogue," *Acta Crystallogr D Biol Crystallogr.*, 64(Pt 3):227-236, (2008).
- Luer and Hatton, "Vancomycin administration into the cerebrospinal fluid: a review," *Annals of Pharmacotherapy*, 27:912-921, (1993).
- Madzak et al., "Strong hybrid promoters and integrative expression/secretion vectors for quasi-constitutive expression of heterologous proteins in the yeast *Yarrowia lipolytica*," *J Mol Microbiol Biotechnol.*, 2(2):207-216, (Apr. 2000).
- Madzak et al., "Heterologous Protein Expression and Secretion in the Non-conventional Yeast *Yarrowia lipolytica*: A Review", *J. Biotechnol.*, 109:63-81 (2004).
- Maras et al., "Molecular cloning and enzymatic characterization of a *Trichoderma reesei* 1, 2-alpha-D-mannosidase," *J. Biotechnol.*, 77: 255-263 (2000).
- Martinet et al., "Protection of mice against a lethal influenza challenge by immunization with yeast-derived recombinant influenza neuraminidase," *Eur J Biochem.*, 247(1):332-338, (Jul. 1997).
- Merkle et al., Cloning, expression, purification, and characterization of the murine lysosomal acid alpha-mannosidase, *Biochim Biophys Acta.*, 1336(2):132-146, (Aug. 1997).
- Moreau et al. "Cell-free transfer of membrane lipids. Evidence for lipid processing," *J. Biol. Chem.* 266(7):4329-4333, (1991).
- Moreau et al. "Trafficking of lipids from the endoplasmic reticulum to the Golgi apparatus in a cell-free system from rat liver," *J. Biol. Chem.*, 266(7):4322-4328, (1991).
- Mori et al., "Signalling from endoplasmic reticulum to nucleus: transcription factor with a basic-leucine zipper motif is required for the unfolded protein-response pathway," *Genes Cells*, vol. 1, No. 9, pp: 803-817, (Sep. 1996).
- Newman and Ferro-Novick, "Characterization of new mutants in the early part of the yeast secretory pathway isolated by a [3H]mannose suicide selection," *J. Cell Biol.*, 105(4):1587-1594, (1987).
- Orlean et al., "Cloning and sequencing of the yeast gene for dolichol phosphate mannose synthase, an essential protein," *J. Biol. Chem.* vol. 263, pp. 17499-17507, (Nov. 1988).
- Paulik et al., "Cell-free transfer of the vesicular stomatitis virus G protein from an endoplasmic reticulum compartment of baby hamster kidney cells to a rat liver Golgi apparatus compartment for Man8-9 to Man5 processing," *Arch. Biochem. Biophys.*, 367(2):265-273, (1999).
- Platt and Lachmann, "Treating lysosomal storage disorders: Current practice and future prospects," *Biochim Biophys Acta*, 1793(4):737-745, (2009).
- Poljak, "Production and structure (1994) of diabodies," *Structure*, 2(12):1121-1123, (1994).
- Protein Data Bank, "Structure of the GH92 Family Glycosylhydrolase CCMAN5" Deposition: Sep. 29, 2010 [retrieved on Jul. 17, 2012]. Retrieved from the Internet: <URL: <http://www.pdb.org/pdb/explore/explore.do?structureId=2XSG>>, 2 pages.
- Rexach and Schekman, "Distinct biochemical requirements for the budding, targeting, and fusion of ER-derived transport vesicles," *J. Cell Biol.*, 114(2):219-229, (1991).
- Richard et al., "Tagging morphogenetic genes by insertional mutagenesis in the yeast *Yarrowia lipolytica*," *J Bacteriol.*, 183(10):3098-3107, (May 2001).
- Smith and Waterman, "Comparison of biosequences," *Adv. Appl. Math.*, 2(4):482-489, (Dec. 1981).
- Song, Y, et al., "Engineering of the Yeast *Yarrowia lipolytica* for the Production of Glycoproteins Lacking the Outer-Chain Mannose Residue of N Glycans," *Applied and Environmental Microbiology*, Jul. 2007, pp. 4446-4454.
- Song et al., "Characterization of Genes Involved in N-glycosylation in *Yarrowia lipolytica*," *Yeast* 20:S147 (2003).
- Sreekrishna et al., "Invertase gene (SUC2) of *Saccharomyces cerevisiae* as a dominant marker for transformation of *Pichia pastoris*," *Gene*, 59(1):115-125, (1987).
- Stocks, "Intrabodies: production and promise," *Drug Discov. Today* 9(22): 960-966, (Nov. 2004).
- Tremblay and Herscovics, "Cloning and expression of a specific human alpha 1,2-mannosidase that trims Man9GlcNAc2 to Man8GlcNAc2 isomer B during N-glycan biosynthesis," *Glycobiology*, 9(10):1073-1078, (Oct. 1999).
- UniProtKB/Swiss-Prot: P06280.1 GI:113499, "RecName: Full=Alpha-galactosidase A; AltName: Full=Alpha-D-galactosidase

(56)

## References Cited

## OTHER PUBLICATIONS

- A; AltName: Full=Alpha-D-galactoside galactohydrolase; AltName: Full=Melibiose; AltName: INN=Agalsidase; Flags: Precursor; Jun. 13, 2012, 26 pages.
- UniProtKB/Swiss-Prot: P15291.5 GI:116241264, "RecName: Full=Beta-1,4-galactosyltransferase 1; Short=Beta-1,4-GalTase 1; Short=Beta4Gal-T1; Short=b4Gal-T1; AltName: Full=UDP-Gal:beta-GlcNAc beta-1,4-galactosyltransferase 1; AltName: Full=UDP-galactose:beta-N-acetylglucosamine beta-1,4-galactosyltransferase . . ." Jun. 13, 2012, 10 pages.
- UniProtKB/Swiss-Prot: P26572.2 GI:311033399, "RecName: Full=Alpha-1,3-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransferase; AltName: Full=N-glycosyl-oligosaccharide-glycoprotein N-acetylglucosaminyltransferase 1; Short=GNT-I; Short=GlcNAc-T I; Apr. 18, 2012, 6 pages.
- UniProtKB/Swiss-Prot: P27809.1 GI:127214, "RecName: Full=Glycolipid 2-alpha-mannosyltransferase; AltName: Full=Alpha-1,2-mannosyltransferase; Jun. 13, 2012, 8 pages.
- UniProtKB/Swiss-Prot: P38069.1 GI:586137, "RecName: Full=Alpha-1,2-mannosyltransferase MNN2; AltName: Full=Calcium resistance and vanadate sensitivity protein 4; AltName: Full=Mannan synthesis protein MNN2; Jun. 13, 2012, 5 pages.
- UniProtKB/Swiss-Prot: Q09326.1 GI:1169978, "RecName: Full=Alpha-1,6-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransferase; AltName: Full=Beta-1,2-N-acetylglucosaminyltransferase II; AltName: Full=GlcNAc-T II; Short=GNT-II; AltName: Full=Mannoside acetylglucosaminyltransferase 2; AltName: Full=N-g . . . , Jun. 13, 2012, 3 pages.
- UniProtKB/Swiss-Prot: Q24451.2 GI:32130434, "RecName: Full=Alpha-mannosidase 2; AltName: Full=Golgi alpha-mannosidase II; Short=AMan II; Short=Man II; AltName: Full=Mannosyl-oligosaccharide 1,3-1,6-alpha-mannosidase; Apr. 18, 2012, 13 pages.
- UniProtKB/Swiss-Prot: Q9Y7X5.1 GI:74698597, "RecName: Full=Uncharacterized protein C365.14c; May 16, 2012, 2 pages.
- Vandersall-Nairn et al., "Cloning, expression, purification, and characterization of the acid  $\alpha$ -mannosidase from *Trypanosoma cruzi*," *Glycobiology*, 8(12):1183-1194, (1998).
- Verostek et al., "Glycoprotein biosynthesis in the alg3 *Saccharomyces cerevisiae* mutant. I. Role of glucose in the initial glycosylation of invertase in the endoplasmic reticulum," *The Journal of Biological Chemistry*, vol. 268, pp. 12095-12103, (Jun. 5, 1993).
- Verostek et al., "Glycoprotein biosynthesis in the alg3 *Saccharomyces cerevisiae* mutant. II. Structure of novel Man6-10GlcNAc2 processing intermediates on secreted invertase," *The Journal of Biological Chemistry*, vol. 268, pp. 12104-12115, (Jun. 5, 1993).
- Vervecken et al., "In Vivo Synthesis of Mammalian-Like, Hybrid-Type N-Glycans in *Pichia pastoris*," *Appl. Environ. Microb.*, 70(5):2639-2646, (May 2004).
- Vocadlo et al., "Mechanistic insights into glycosidase chemistry," *Curr. Opin. Chem. Biol.* 12:539-555 (2008).
- Ward et al., "Characterization of Humanized Antibodies Secreted by *Aspergillus niger*," *Appl. Environ. Microbiol.*, 70(5):2567-2576, (May 2004).
- Wheeler et al., "Intrabody and Intrakine Strategies for Molecular Therapy," *Mol. Ther.*, 8(3):355-366, (Sep. 2003).
- Yang et al., "Cell-surface display of the active mannanase in *Yarrowia lipolytica* with a novel surface-display system," *Biotechnol Appl Biochem*, vol. 54, No. 3, pp. 171-176, (Oct. 2009).
- Yue et al., "Construction of a new plasmid for surface display on cells of *Yarrowia lipolytica*," *J Microbiol Methods*, vol. 72, No. 2, pp. 116-23, (Feb. 2008).
- Zhu and Zhang, "SCPD: a promotor database of the yeast *Saccharomyces cerevisiae*," *Bioinformatics*, 15(7-8):607-611, (1999).
- Zhu et al., "Mechanistic insights into a Ca<sup>2+</sup>-dependent family of alpha-mannosidases in a human gut symbiont," *Nat. Chem. Biol.*, 6(2):125-132. Epub Dec. 27, 2009 (2010).
- Zimm et al., "Cerebrospinal fluid pharmacokinetics of intraventricular and intravenous aziridinylbenzoquinone," *Cancer Research*, 44(4):1698-1701, (Apr. 1984)
- International Search Report and Written Opinion in PCT/IB2008/001814, mailed Mar. 25, 2009, 31 pages.
- Aravind and Koonin, "The fukutin family—predicted enzymes modifying cell-surface molecules," *Curr Biol.*, 9(22):R836-R837, Nov. 18, 1999.
- Bijvoet et al., "Recombinant human acid alpha-glucosidase: high level production in mouse milk, biochemical characteristics, correction of enzyme deficiency in GSDII KO mice," *Hum Mol Genet.*, 7(11):1815-1824, Oct. 1998.
- Bourbonnais et al., "Production of full-length human pre-elafin, an elastase specific inhibitor, from yeast requires the absence of a functional yapsin 1 (Yps1p) endoprotease," *Protein Expr Purif.*, 20(3):485-491, Dec. 2000.
- Cardone et al., "Abnormal mannose-6-phosphate receptor trafficking impairs recombinant alpha-glucosidase uptake in Pompe disease fibroblasts," *Pathogenetics*, 1(1):6, Dec. 1, 2008.
- De Pourcq et al., "Engineering of glycosylation in yeast and other fungi: current state and perspectives," *Appl Microbiol Biotechnol.*, 87(5):1617-1631. Epub Jun. 29, 2010.
- De Pourcq et al., "Engineering the yeast *Yarrowia lipolytica* for the production of therapeutic proteins homogeneously glycosylated with Man8GlcNAc2 and MansGlcNAc2," *Microbial Cell Factories*, 11:53, 1-12, May 1, 2012.
- Ettinger et al., "Intrathecal methotrexate overdose without neurotoxicity: case report and literature review," *Cancer*, 41(4):1270-1273, Apr. 1978.
- Gagnon-Arsenault et al., "Activation mechanism, functional role and shedding glycosylphosphatidylinositol-anchored Yps1p at the *Saccharomyces cerevisiae* of cell surface," *Mol Microbiol.*, 69(4):982-993, Epub Jun. 28, 2008.
- Gagnon-Arsenault et al., "Fungal yapsins and cell wall: a unique family of aspartic peptidases for a distinctive cellular function," *FEMS Yeast Res.*, 6(7):966-978, Nov. 2006.
- GenBank Accession No. XP\_503768, GI: 50552716, "YALI0E10175p [*Yarrowia lipolytica* CLIB122]," Oct. 29, 2008, 2 pages.
- Inoue et al., "Molecular cloning and nucleotide sequence of the 1,2-alpha-D-mannosidase gene, msdS, from *Aspergillus saitoi* and expression of the gene in yeast cells," *Biochim Biophys Acta*. 1253(2):141-145, Dec. 6, 1995.
- Jacobs et al., "Engineering complex-type N-glycosylation in *Pichia pastoris* using GlycoSwitch technology," *Nat Protoc.*, 2009;4(1):58-70., Epub Dec. 18, 2008.
- Komeda et al., "Construction of protease-deficient *Candida boidinii* strains useful for recombinant protein production: cloning and disruption of proteinase A gene (PEP4) and proteinase B gene (PRB1)," *Biosci Biotechnol Biochem.*, 66(3):628-631, Mar. 2002.
- Kuroda et al., "Antibody expression in protease-deficient strains of the methylotrophic yeast *Ogataea minuta*," *FEMS Yeast Res.*, 7(8):1307-1316. Epub Aug. 22, 2007.
- Le Dall et al., "Multiple-copy integration in the yeast *Yarrowia lipolytica*," *Curr Genet.*, 26(1):38-44, Jul. 1994.
- Li et al., "Optimization of humanized IgGs in glycoengineered *Pichia pastoris*," *Nat Biotechnol.*, 24(2):210-215, Epub Jan. 22, 2006.
- Liu et al., "Disruption of the OCH1 and MNN1 genes decrease N-glycosylation on glycoprotein expressed in *Kluyveromyces fragilis*," *J Biotechnol.*, 143(2):95-102, Epub Jun. 24, 2009.
- Mille et al., "Identification of a new family of genes involved in beta-1,2-mannosylation of glycans in *Pichia pastoris* and *Candida albicans*," *J Biol Chem.*, 283(15):9724-9736. Epub Jan. 30, 2008.
- Moreland et al., "Lysosomal acid alpha-glucosidase consists of four different peptides processed from a single chain precursor," *J Biol Chem.*, 280(8):6780-6791, Epub Nov. 1, 2004.
- Nakadai et al., "Purification and Properties of Alkaline Proteinase from *Aspergillus oryzae*," *Agri. Biol. Chem.*, 37(12): 2685-2694, 1973.

(56)

## References Cited

## OTHER PUBLICATIONS

- NCBI-GenBank, "Yarrowia lipolytica CLIB122 [gbpln]: 5967 CDS's (2945919 codons)," Codon Usage Database, [online], Jun. 15, 2007 [retrieved on Aug. 15, 2014]. Retrieved from the Internet: <URL: <http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=284591>>, 1 page.
- Peberdy et al., "Protein secretion by fungi," *Applied Microbiology and Biotechnology, Agriculture and Food Production*, 1:73-114, 2001.
- Penttilä et al., "Expression of two Trichoderma reesei endoglucanases in the yeast *Saccharomyces cerevisiae*," *Yeast*, 3(3):175-185, Sep. 1987.
- Potgieter et al., "Production of monoclonal antibodies by glycoengineered *Pichia pastoris*," *J Biotechnol.*, Feb. 23, 2009;139(4):318-325, Epub Dec. 27, 2008.
- Seffernick et al., "Melamine deaminase and atrazine chlorohydrolase: 98 percent identical but functionally different," *J Bacteriol.*, 183(8):2405-2410, Apr. 2001.
- Swennen et al., "Folding proteome of *Yarrowia lipolytica* targeting with uracil permease mutants," *J Proteome Res.*, 9(12):6169-6179, Epub Nov. 12, 2010.
- Swiss Protein Accession No. P15291, Nov. 30, 2010, 9 pages.
- Swiss Protein Accession No. P26572, Nov. 30, 2010, 4 pages.
- Swiss Protein Accession No. P38069, Nov. 30, 2010, 3 pages.
- Swiss Protein Accession No. Q09326, Nov. 30, 2010, 3 pages.
- Swiss Protein Accession No. Q24451, Nov. 30, 2010, 12 pages.
- Tiels et al., "A bacterial glycosidase enables mannose-6-phosphate modification and improved cellular uptake of yeast-produced recombinant human lysosomal enzymes," *Nat Biotechnol.*, 30(12):1225-1231, Epub Nov. 18, 2012.
- Van Hove et al., "High-level production of recombinant human lysosomal acid alpha-glucosidase in Chinese hamster ovary cells which targets to heart muscle and corrects glycogen accumulation in fibroblasts from patients with Pompe disease," *Proc Natl Acad Sci U S A.*, 93(1):65-70, Jan. 9, 1996.
- Vervecken et al., "Modification of the N-glycosylation pathway to produce homogeneous, humanlike glycans using GlycoSwitch plasmids," *Methods Mol Biol.*, 389:119-138, 2007.
- Witkowski et al., "Conversion of a beta-ketoacyl synthase to a malonyl decarboxylase by replacement of the active-site cysteine with glutamine," *Biochemistry*, 38(36):11643-11650, Sep. 7, 1999.
- Wu et al., "Asparagine-linked glycosylational modifications in yeast," *Cell Engineering*, 3:215-232, 2002.
- YALIOA16819g YALIOA16819p[Yarrowia lipolytica CLIB122] Gene ID: 2906333, created on Jul. 24, 2004, 2 pages.
- YALIOC10135g YALIOC10135p[Yarrowia lipolytica CLIB122] Gene ID: 7009445, created on Oct. 29, 2008, 2 pages.
- YALIOD10835g YALIOD10835p[Yarrowia lipolytica CLIB122] Gene ID: 2910442, created on Jul. 24, 2004, 2 pages.
- YALIOE10175g YALIOE10175p[Yarrowia lipolytica CLIB122] Gene ID: 2912589, created on Jul. 28, 2004, 2 pages.
- YALIOE20823g YALIOE20823p[Yarrowia lipolytica CLIB122] Gene ID: 2911836, created on Jul. 28, 2004, 2 pages.
- YALIOE22374g YALIOE22374p[Yarrowia lipolytica CLIB122] Gene ID: 2912981, created on Jul. 28, 2004, 2 pages.
- YALIOE34331g YALIOE34331p[Yarrowia lipolytica CLIB122] Gene ID: 2912367, created on Jul. 28, 2004, 2 pages.
- YALIOE24981g YALIOE24981p[Yarrowia lipolytica CLIB122] Gene ID: 2912672, created on Jul. 28, 2004, 2 pages.
- Yao et al., "Degradation of HSA-AX15(R13K) when expressed in *Pichia pastoris* can be reduced via the disruption of YPS1 gene in this yeast," *J Biotechnol.*, Jan. 15, 2009;139(2):131-136. Epub Oct. 8, 2008.
- Zhu et al., "Glycoengineered acid alpha-glucosidase with improved efficacy at correcting the metabolic aberrations and motor function deficits in a mouse model of Pompe disease," *Mol Ther.*, 17(6):954-963, Epub Mar. 10, 2009.
- Search Report issued in SG Application No. 201109764-9, mailed Apr. 30, 2014, 20 pages.
- Hamilton et al; Production of complex human glycoproteins in yeast; Science; Aug. 2003; 301(5637):1244-1246.
- Choi, et al., "Structural analysis of N-linked oligosaccharides assembled on recombinant proteins secreted from *Yarrowia lipolytica* Yloch1 and Yloch1 Ylmnn4 mutants," XXIIth International Conference on Yeast Genetics and Molecular Biology, 09—Protein biosynthesis, maturation, modification and degradation, Yeast, 22:S131, 9-35, 2005.
- "Glycoside Hydrolase Family 38," cazy.org [online] captured Sep. 11, 2010. Retrieved from the Internet: <URL: <http://www.cazy.org/GH38.html>>, 1 page.
- "Glycoside Hydrolase Family 47," cazy.org [online] captured Sep. 12, 2010. Retrieved from the Internet: <URL: <http://www.cazy.org/GH47.html>>, 1 page.
- "Glycoside Hydrolase Family 92," cazy.org [online] captured Sep. 12, 2010. Retrieved from the Internet: <URL: <http://www.cazy.org/GH92.html>>, 1 page.
- Cantarel et al., "The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics," *Nucleic Acids Res.*, 37(Database issue):D233-D238, Epub Oct. 5, 2008.
- Gatlin et al., "Automated identification of amino acid sequence variations in proteins by HPLC/microspray tandem mass spectrometry," *Anal Chem.*, 72(4):757-763, Feb. 15, 2000.
- Gilbert, "Glycoside Hydrolase Family 92," CAZypedia [online], Mar. 4, 2010. Retrieved from the Internet: <URL: [http://www.cazypedia.org/index.php/Glycoside\\_Hydrolase\\_Family\\_92](http://www.cazypedia.org/index.php/Glycoside_Hydrolase_Family_92)>, 3 pages.
- Henrissat, "A classification of glycosyl hydrolases based on amino acid sequence similarities," *Biochem J.*, 280 (Pt 2):309-316, Dec. 1, 1991.
- Mast and Moremen, "Family 47 alpha-mannosidases in N-glycan processing," *Methods Enzymol.*, 415:31-46, 2006.
- Rose, "Glycoside Hydrolase Family 38," CAZypedia [online], Feb. 2, 2010. Retrieved from the Internet: <URL: [http://www.cazypedia.org/index.php/Glycoside\\_Hydrolase\\_Family\\_38](http://www.cazypedia.org/index.php/Glycoside_Hydrolase_Family_38)>, 3 pages.
- Van den Elsen et al., "Structure of Golgi alpha-mannosidase II: a target for inhibition of growth and metastasis of cancer cells," *EMBO J.*, 20(12):3008-3017, Jun. 15, 2001.
- Abe et al., "In vitro oligosaccharide synthesis using intact yeast cells that display glycosyltransferases at the cell surface through cell wall-anchored protein Pir," *Glycobiology*, 13(2):87-95, print Feb. 2003, epub Nov. 2002.
- Ackerman et al., "Highly avid magnetic bead capture: an efficient selection method for de novo protein engineering utilizing yeast surface display," *Biotechnol Prog.*, 25(3):774-783, May-Jun. 2009.
- Almeciga et al., "Production of an active recombinant human N-acetylgalactosamine-6-sulfate sulfatase enzyme in *Pichia pastoris*," *Molecular Genetics and Metabolism*, 111(2):S19, Abstract 11, Jan. 27, 2014.
- Andrés et al., "Use of the cell wall protein Pir4 as a fusion partner for the expression of *Bacillus* sp. BP-7 xylanase A in *Saccharomyces cerevisiae*," *Biotechnol Bioeng*, 89(6): 690-697, Mar. 2005.
- Boder and Wittrup, "Yeast surface display for screening combinatorial polypeptide libraries," *Nat. Biotechnol.*, 15, 553-557, Jun. 1997.
- Boder et al., "Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity," *Proc Natl Acad Sci U S A.*, 97(20):10701-5, Sep. 2000.
- Brady, "Enzyme replacement for lysosomal diseases," *Annu. Rev. Med.*, 57:283-296, 2006.
- Brady, "The lipid storage diseases: new concepts and control," *Ann Intern Med.*, 82(2):257-61, Feb. 1975.
- Carlson et al., "Function and structure of a prokaryotic formylglycine-generating enzyme," *J Biol Chem.*, 283(29):20117-20125, Epub Apr. 4, 2008.
- Chao et al., "Isolating and engineering human antibodies using yeast surface display," *Nat. Protoc.*, 1(2):755-768, 2006.
- Chiba et al., "Production of human compatible high mannose-type (Man5GlcNAc2) sugar chains in *Saccharomyces cerevisiae*," *J Biol Chem.*, 273(41):26298-26304, Oct. 9, 1998.
- Cho et al., "Structure of the extracellular region of HER2 alone and in complex with the Herceptin," *Nature*, 421(6924):756-760, Feb. 2003.
- Davidow et al., "Cloning and sequencing of the alkaline extracellular protease gene of *Yarrowia lipolytica*," *J. Bacteriol.*, 169(10):4621-4629, Oct. 1987.

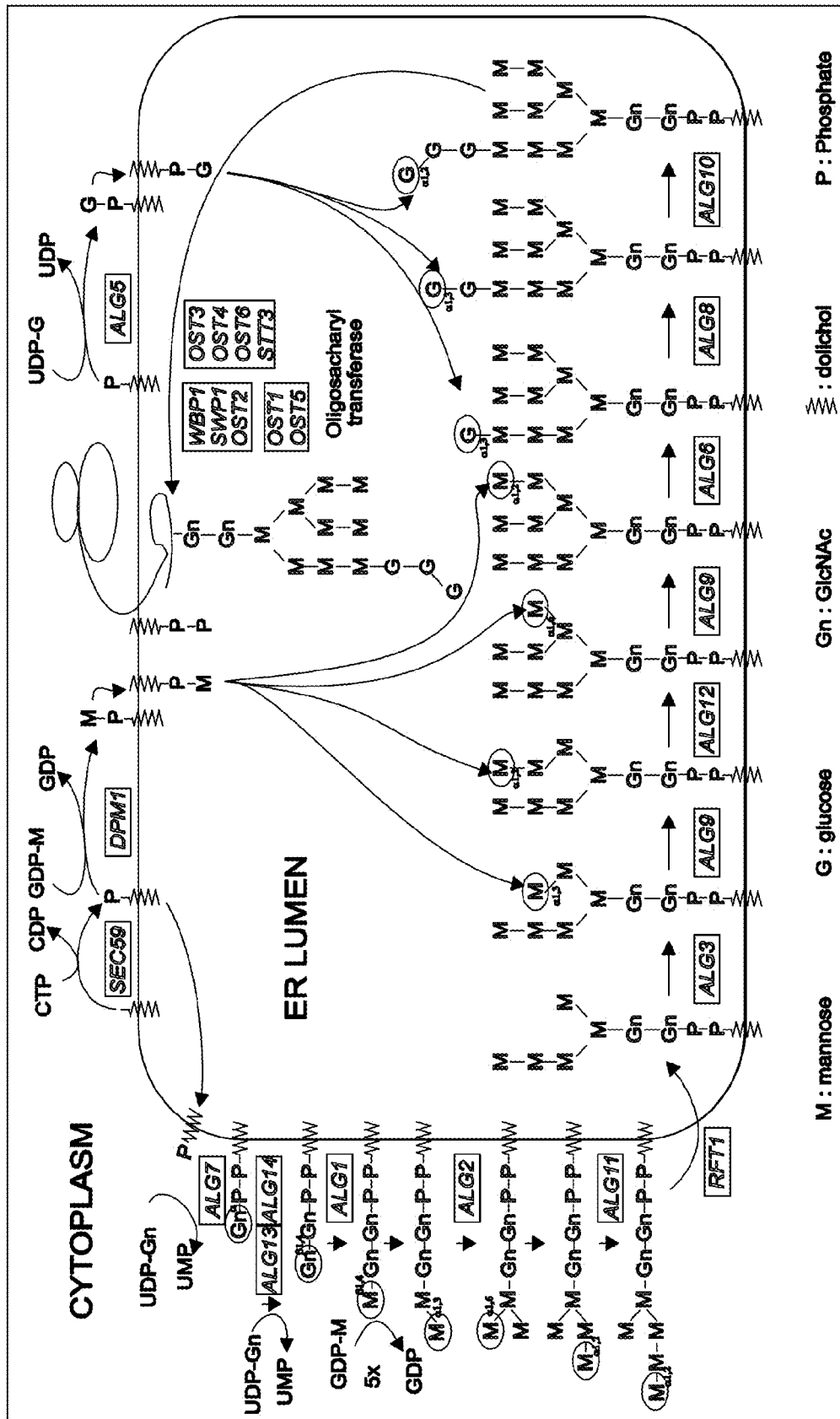
(56)

## References Cited

## OTHER PUBLICATIONS

- Devos and Valencia, "Practical limits of function prediction," *Proteins*, 41(1):98-107, Oct. 1, 2000.
- Dragosits et al., "The effect of temperature on the proteome of recombinant *Pichia pastoris*," *J. Proteome Res.*, 8(3):1380-1392, Mar. 2009.
- Fournier et al., "Scarcity of ars sequences isolated in a morphogenesis mutant of the yeast *Yarrowia lipolytica*," *Yeast*, 7(1):25-36, Jan. 1991.
- Fujii, "Antibody Affinity Maturation by Random Mutagenesis," *Antibody Engineering*, vol. 248, pp. 345-359, 2004.
- Gasser et al., "Engineering of *Pichia pastoris* for improved production of antibody fragments," *Biotechnol. Bioeng.*, 94(2):353-361, Jun. 2006.
- GenBank Accession No. AAO78636, "putative alpha-1,2-mannosidase [Bacteroides thetaiotaomicron VPI-5482]," 1 page, Oct. 24, 2007.
- GenBank Accession No. NP\_630514, "secreted protein [Streptomyces coelicolor A3(2)]," 2 pages, Sep. 26, 2008.
- Grubb et al., "New strategies for enzyme replacement therapy for lysosomal storage diseases," *Rejuvenation Res.*, 13(2-3):229-236, Apr.-Jun. 2010.
- Jaafar et al., "Isolation of the MNN9 gene of *Yarrowia lipolytica* (Y1MNN9) and phenotype analysis of a mutant ylmnn9 Delta strain," *Yeast*, 20(7):633-644, May 2003.
- Klis et al., "Cell wall construction in *Saccharomyces cerevisiae*," *Yeast*, 23(3):185-202, 2006.
- Landgrebe et al., "The human SUMF1 gene, required for post-translational sulfatase modification, defines a new gene family which is conserved from pro- to eukaryotes," *Gene*, 316:47-56, Oct. 16, 2003.
- Lin et al., "Display of a functional hetero-oligomeric catalytic antibody on the yeast cell surface," *App. Microbiol Biotechnol.*, 62(2-3):226-232, print Aug. 2003, Epub Mar. 2003.
- Matsuoka et al., "Analysis of regions essential for the function of chromosomal replicator sequences from *Yarrowia lipolytica*," *Mol. Gen. Genet.*, 237(3):327-333, Mar. 1993.
- Nicaud et al., "Protein expression and secretion in the yeast *Yarrowia lipolytica*," *FEMS Yeast Res.*, 2(3):371-379, Aug. 2002.
- Pignède et al., "Characterization of an extracellular lipase encoded by LIP2 in *Yarrowia lipolytica*," *J. Bacteriol.*, 182(10):2802-10, May 2000.
- Rakestraw and Wittrup, "Contrasting secretory processing of simultaneously expressed heterologous proteins in *Saccharomyces cerevisiae*," *Biotechnol. Bioeng.*, 93(5):896-905, Apr. 2006.
- Rodriguez et al., "Production of recombinant human N-acetylgalactosamine-6-sulfate sulfatase enzyme in *Pichia pastoris*," *Molecular Genetics and Metabolism*, 108(2):579-580, Abstract 197, Feb. 1, 2013.
- Ruiz-Herrera and Sentandreu, "Different effectors of dimorphism in *Yarrowia lipolytica*," *Arch. Microbiol.*, 178(6): 477-483, print Dec. 2002, Epub Oct. 2002.
- Ryckaert et al., "Isolation of antigen-binding camelid heavy chain antibody fragments (nanobodies) from an immune library displayed on the surface of *Pichia pastoris*," *J Biotechnol.*, 145(2):93-98, Epub Oct. 2009, print Jan. 2010.
- Shusta et al., "Increasing the secretory capacity of *Saccharomyces cerevisiae* for production of single-chain antibody fragments," *Nat. Biotechnol.*, 16(8): 773-777, Aug. 1998.
- Shusta et al., "Yeast polypeptide fusion surface display levels predict thermal stability and soluble secretion efficiency," *J. Mol. Biol.*, 292(5):949-956, Oct. 1999.
- Swennen et al., "Secretion of active anti-Ras single-chain Fv antibody by the yeasts *Yarrowia lipolytica* and *Kluyveromyces lactis*," *Microbiology*, 148(Pt 1):41-50, Jan. 2002.
- Tajima et al., "Use of a modified alpha-N-acetylgalactosaminidase in the development of enzyme replacement therapy for Fabry disease," *Am J Hum Genet.*, 85(5):569-580 Epub Oct. 22, 2009.
- Tanino et al., "Construction of a *Pichia pastoris* cell-surface display system using Flo1p anchor system," *Biotechnol. Prog.*, 22(4): 989-993, Jul.-Aug. 2006.
- Ueda et al., "Cell surface engineering of yeast: construction of arming yeast with biocatalyst," *J. Biosci. Bioeng.*, 90(2): 125-136, 2000.
- VanAntwerp and Wittrup, "Fine affinity discrimination by yeast surface display and flow cytometry," *Biotechnol. Prog.*, 16(1): 31-7, Jan.-Feb. 2000.
- Vernis et al., "An origin of replication and a centromere are both needed to establish a replicative plasmid in the yeast *Yarrowia lipolytica*," *Mol. Cell Biol.*, 17(4): 1995-2004, Apr. 1997.
- Wang and Shusta, "The use of scFv-displaying yeast in mammalian cell surface selections," *J. Immunol. Methods*, 304(1-2):30-42, Sep. 2005.
- Wang et al., "A new yeast display vector permitting free scFv amino termini can augment ligand binding affinities," *Protein Eng. Des. Sel.*, 18(7): 337-343, print Jul. 2005, Epub Jun. 2005.
- Wang et al., "Construction of a novel *Pichia pastoris* cell-surface display system based on the cell wall protein Pirl," *Curr. Microbiol.*, 56(4): 352-357, Apr. 2008.
- Whisstock and Lesk, "Prediction of protein function from protein sequence and structure," *Q Rev Biophys.*, 36(3):307-340, Aug. 2003.
- Yeung and Wittrup, "Quantitative screening of yeast surface-displayed polypeptide libraries by magnetic bead capture," *Biotechnol. Prog.*, 18(2):212-220, Mar.-Apr. 2002.
- Ying et al., "Soluble monomeric IgG1 Fc," *J Biol Chem.*, 287(23):19399-19408, Epub Apr. 19, 2012.
- Partial European Search Report; Mar. 26, 2013; European Patent Office (EPO); 12000268.8; 7 pages.
- Akcapinar et al; Effect of codon optimization on the expression of *Trichoderma reesei* endoglucanase 1 in *Pichia pastoris*; *Biotechnol Prog*; epub Jul. 2011; 27(5):1257-1263; doi: 10.1002/btpr.663.
- Choe-Min-Hui; Structural analysis of N-linked oligosaccharides assembled on recombinant proteins secreted from *Yarrowia lipolytica* Yloch1 and Yloch1 Ylmnn4 mutants; Thesis, Chungnam National University; Department of Microbiology; 2006; 39 pages; Republic of Korea.

\* cited by examiner



16E

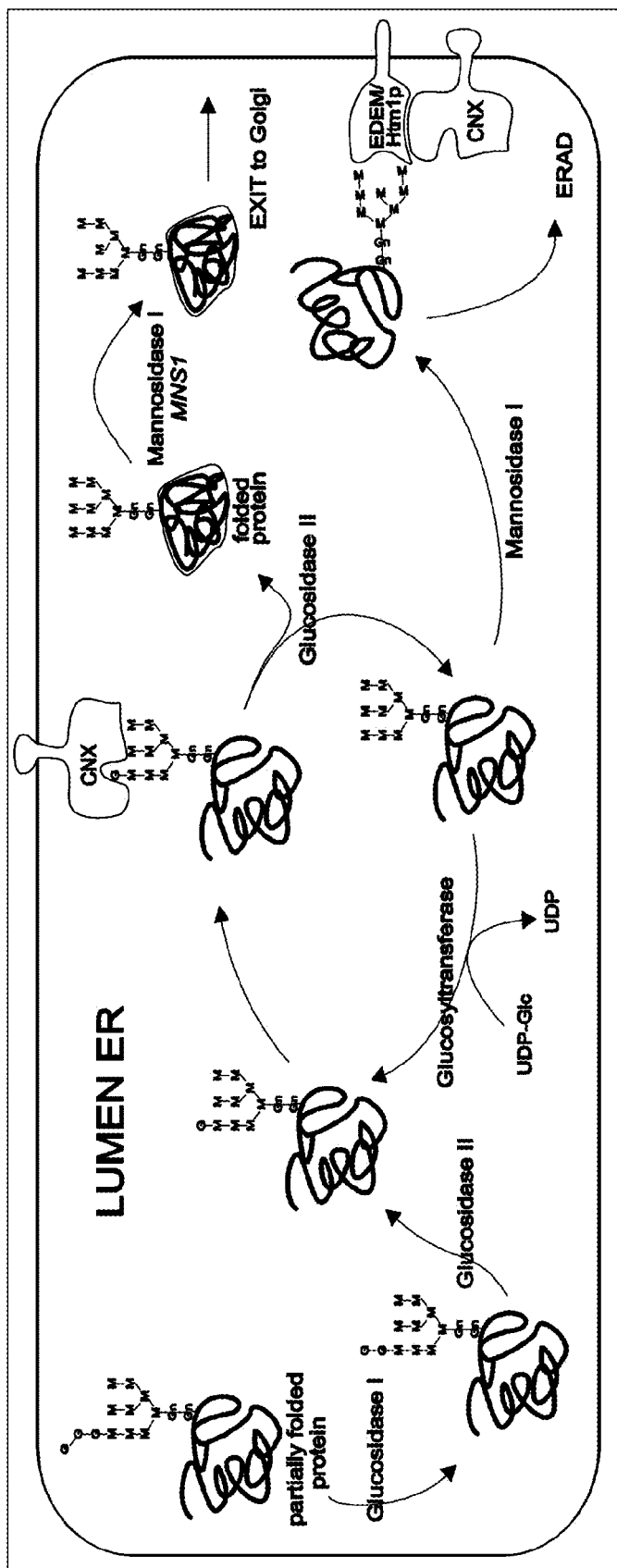


FIG. 2

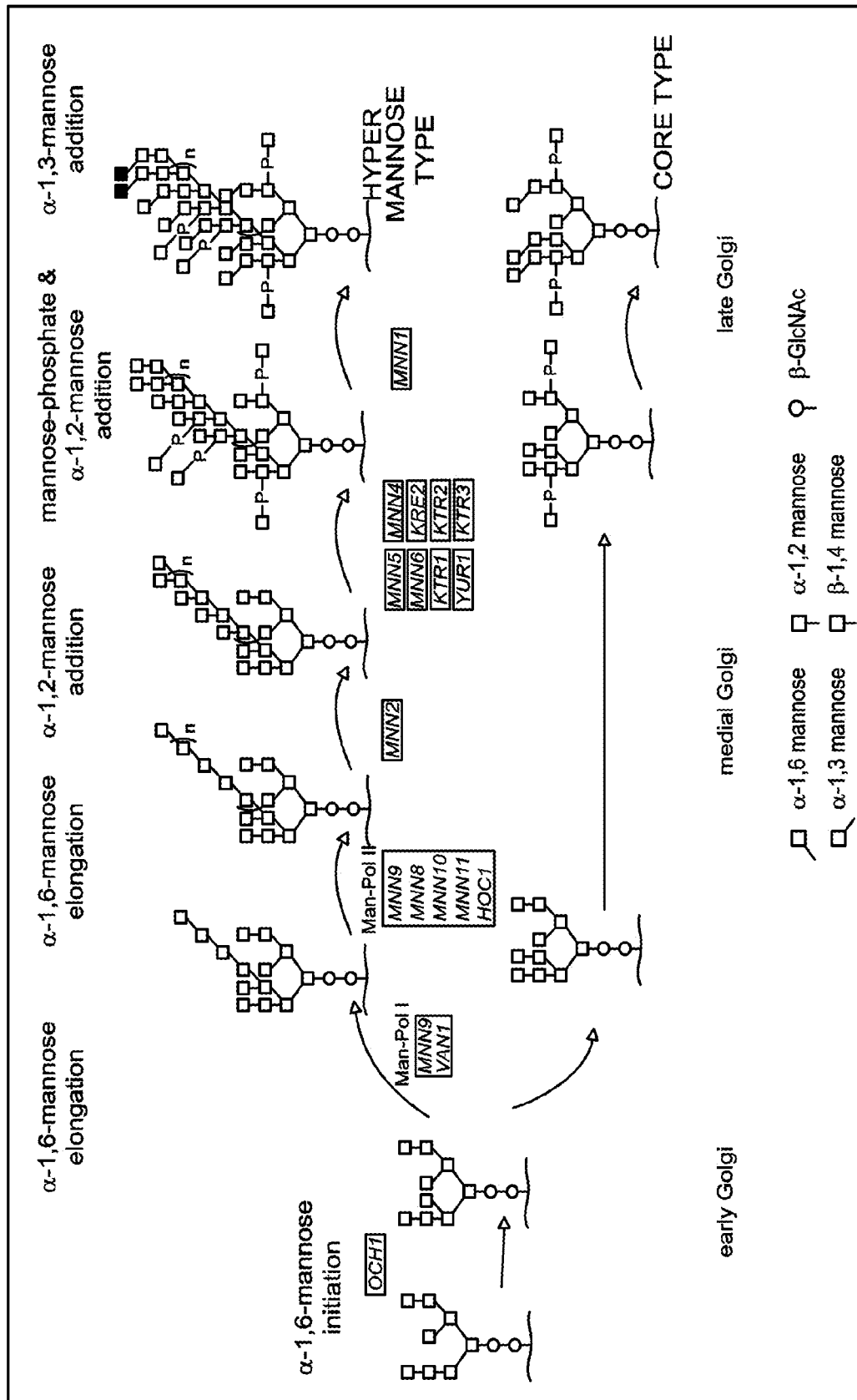


FIG. 3

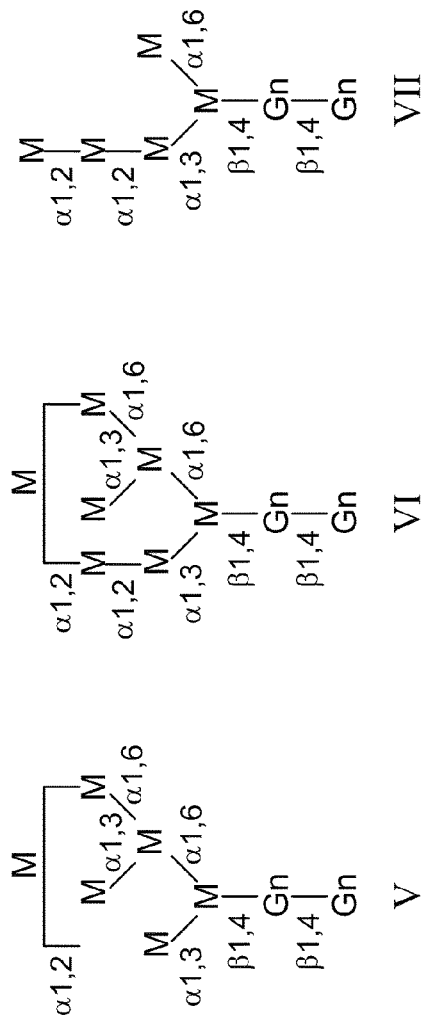
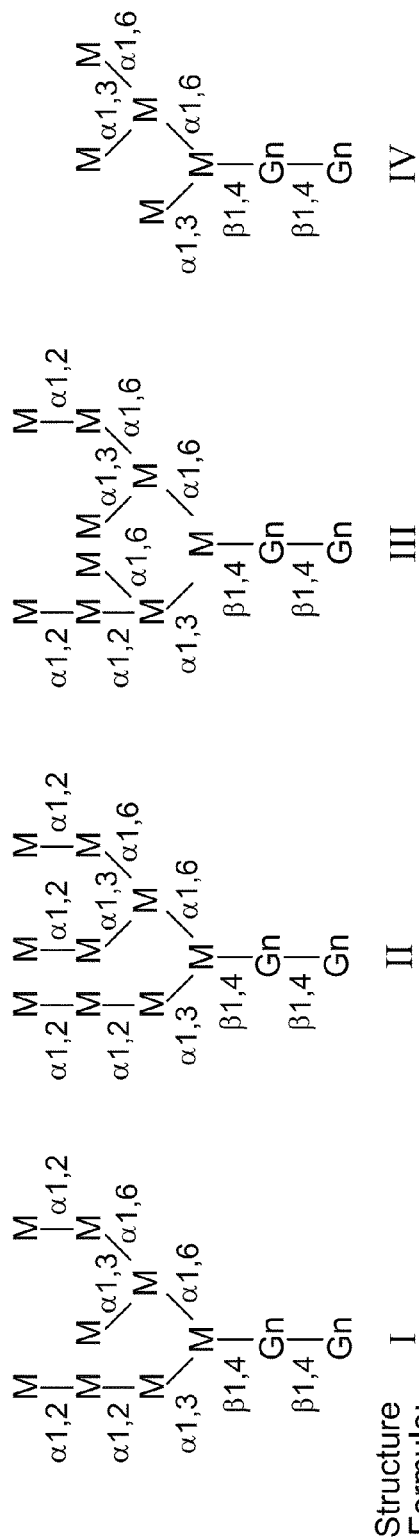
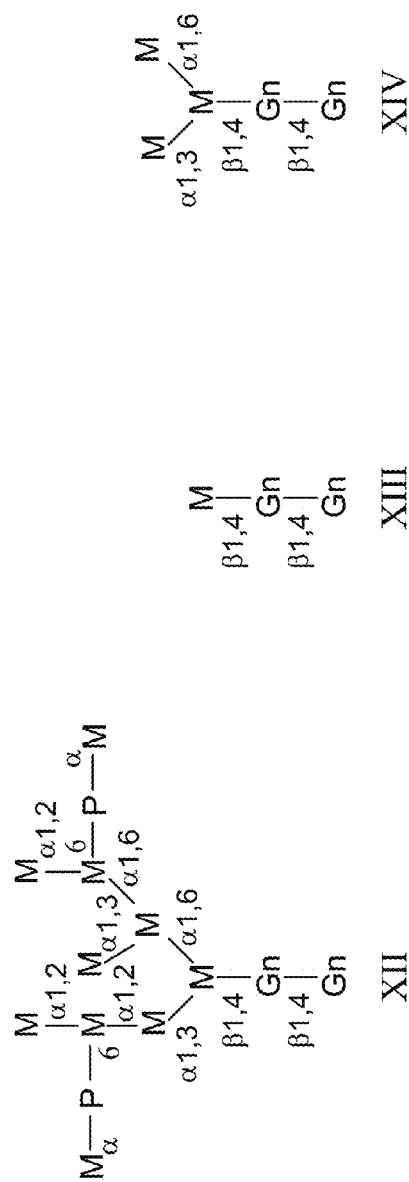
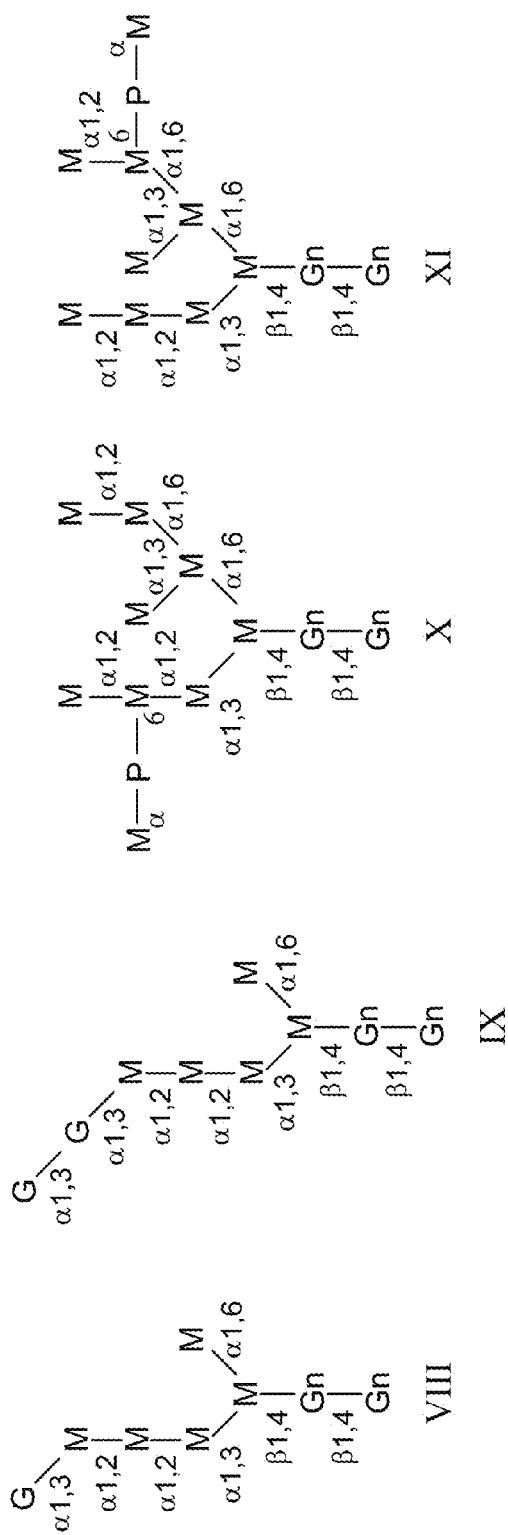


FIG. 4A



M: Mannose

G: Glucose

Gn: N-acetylglucosamine

P: Phosphate

FIG. 4B

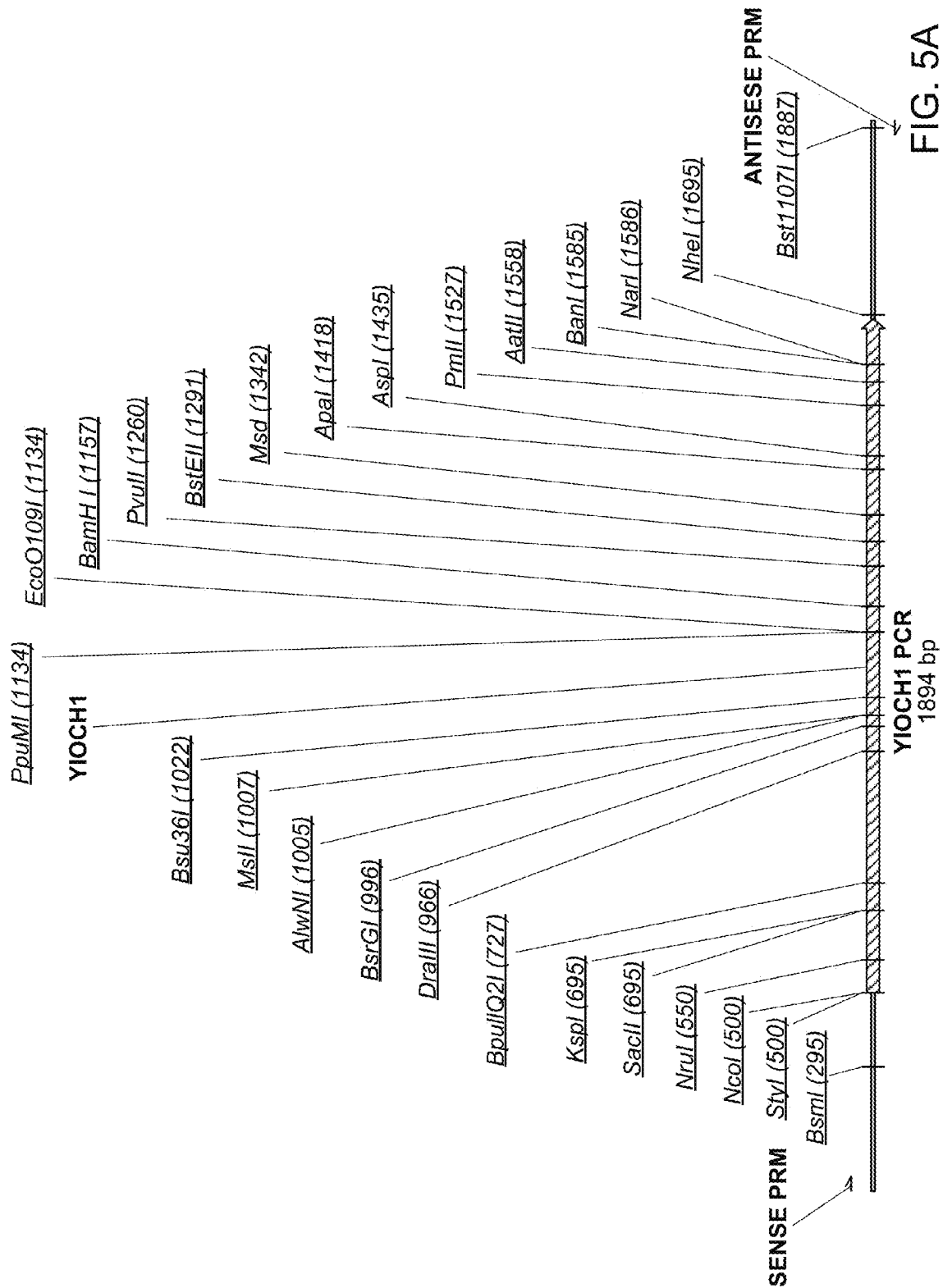


FIG. 5A

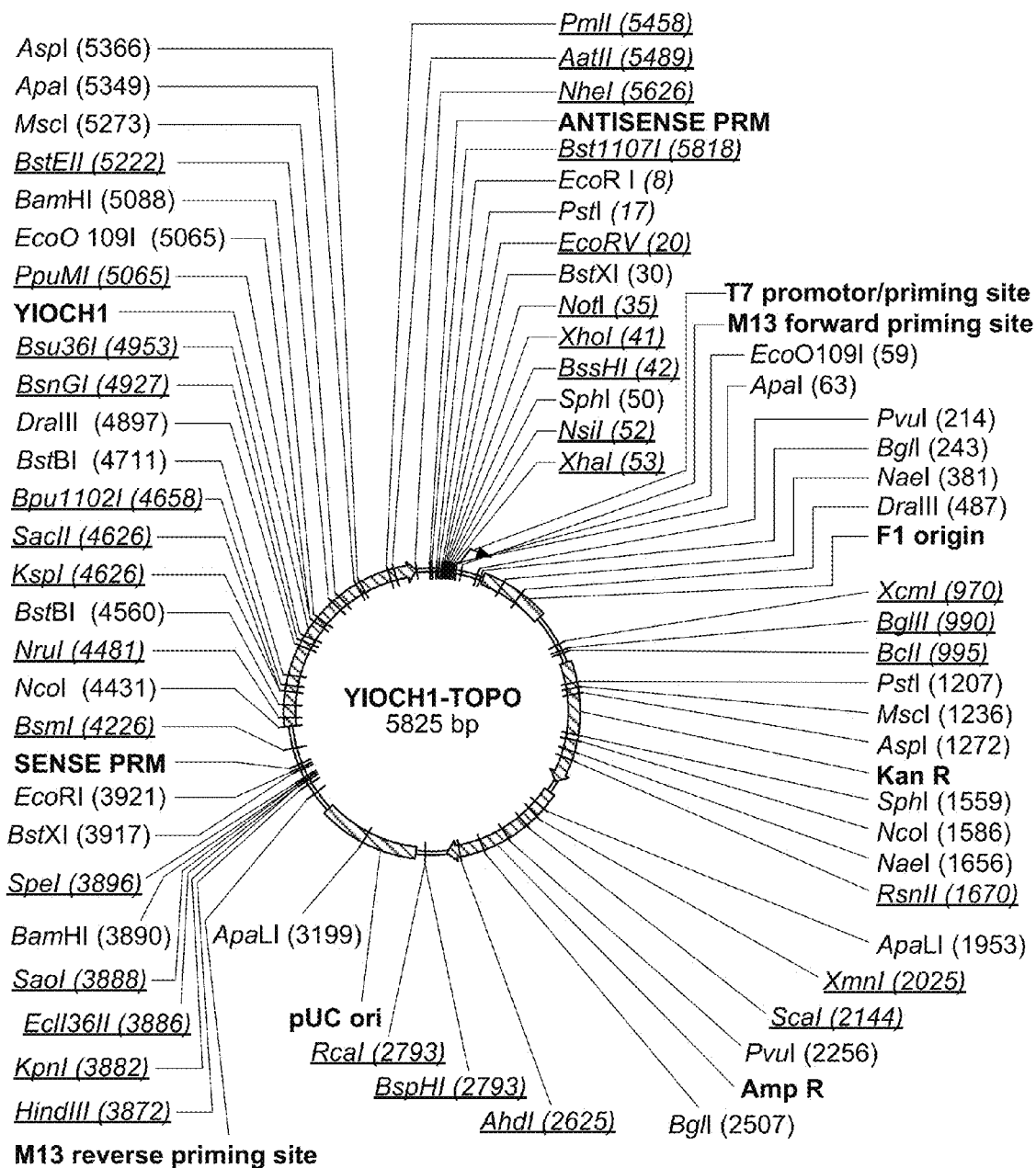
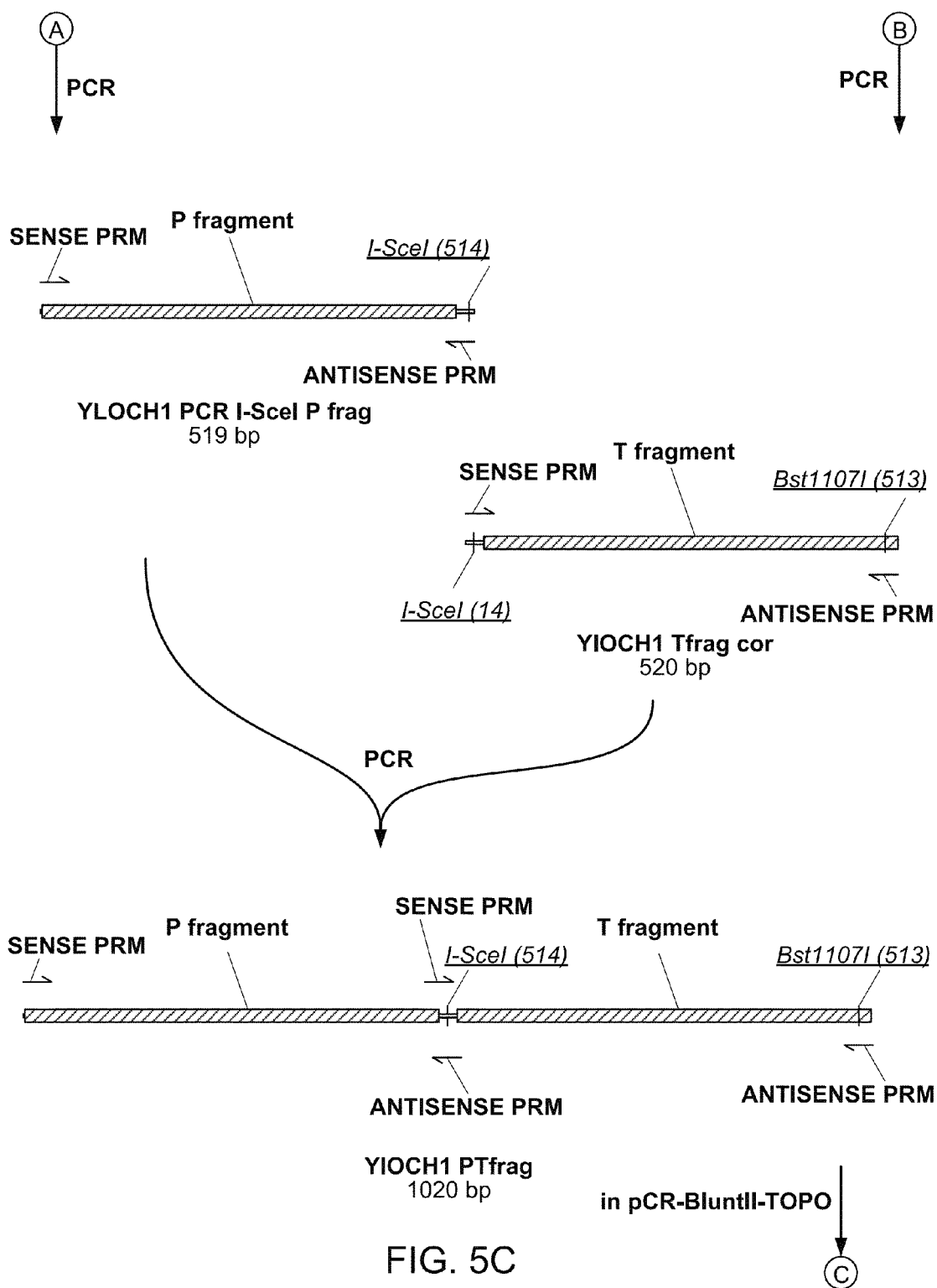


FIG. 5B



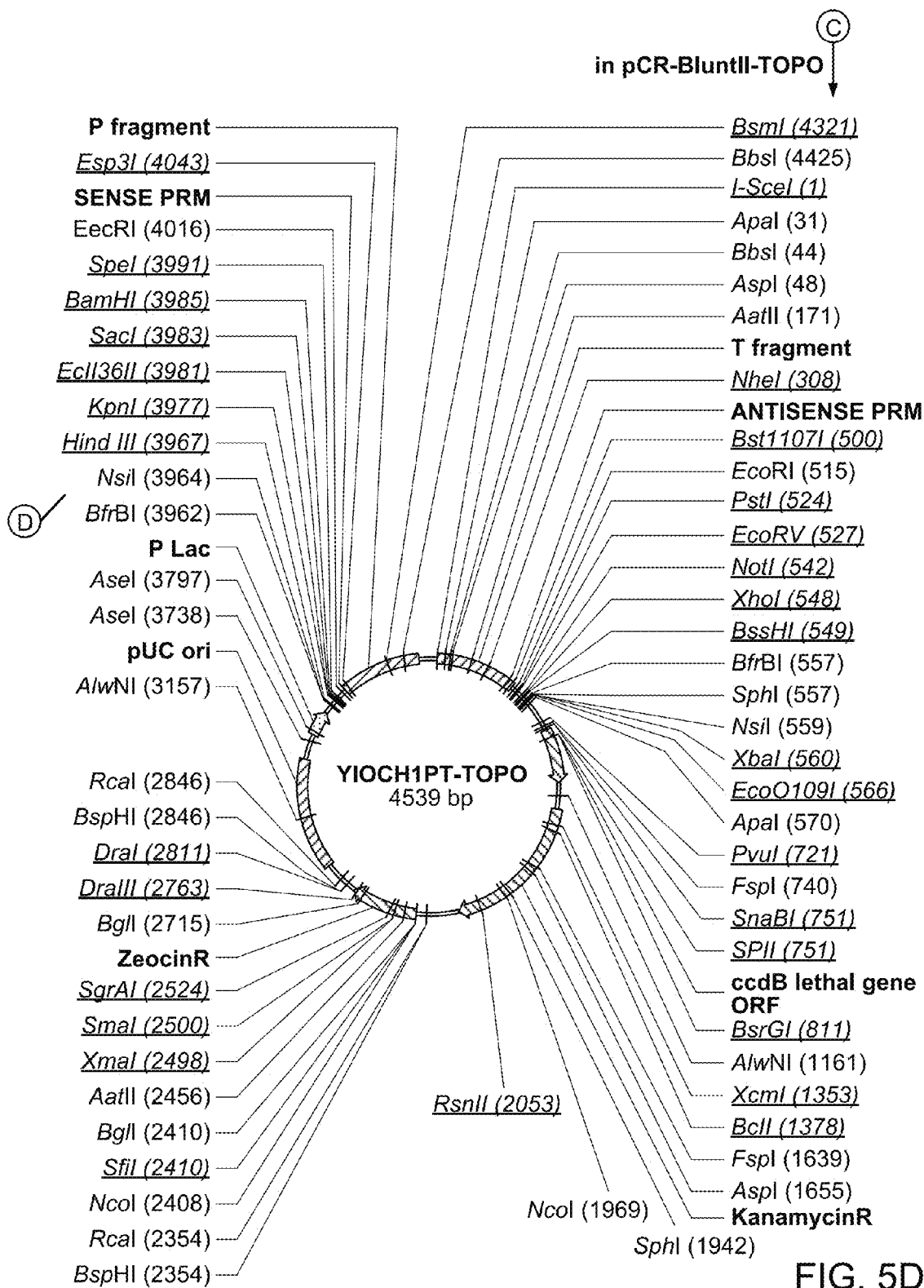


FIG. 5D

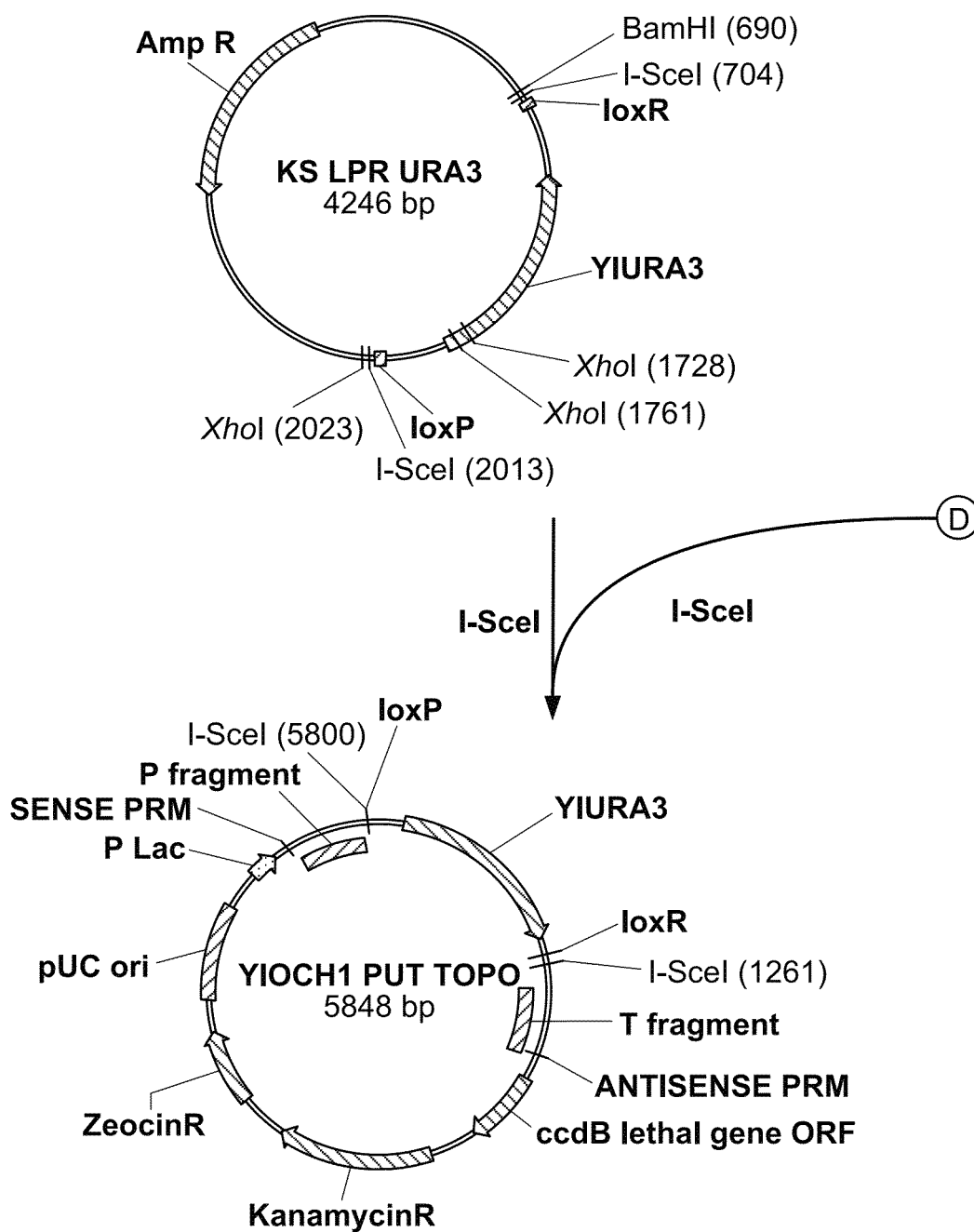


FIG. 5E

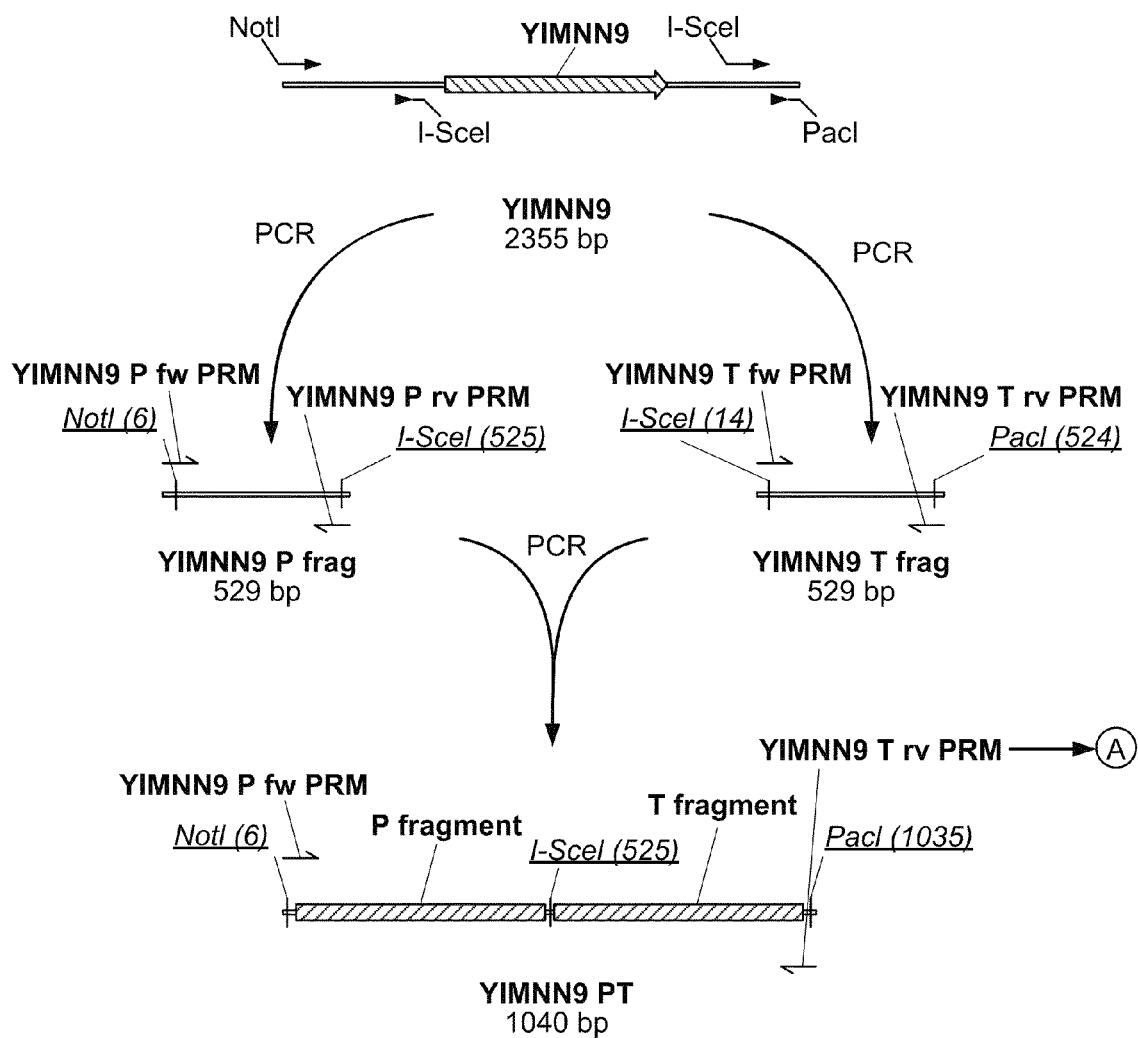


FIG. 6A

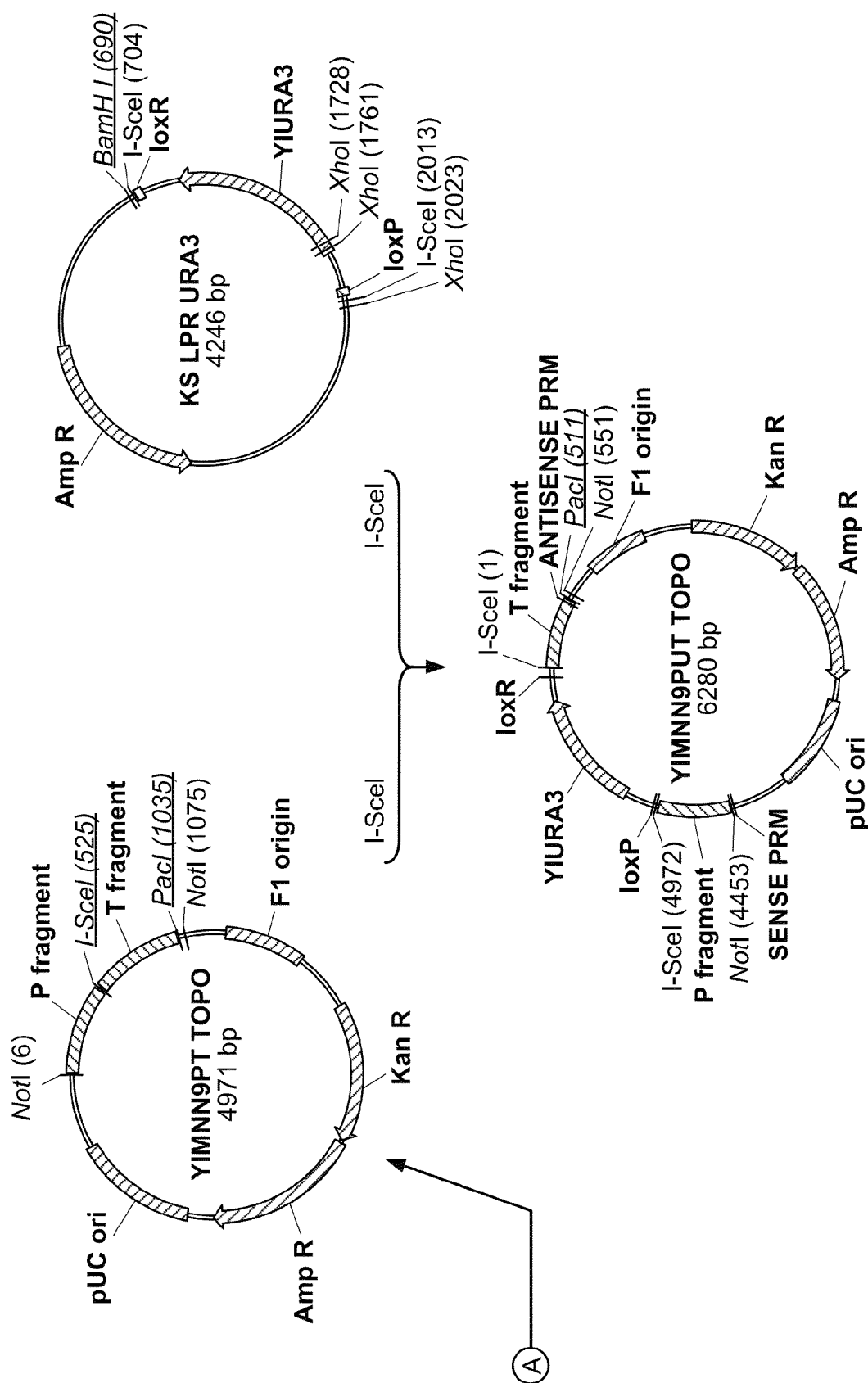


FIG. 6B

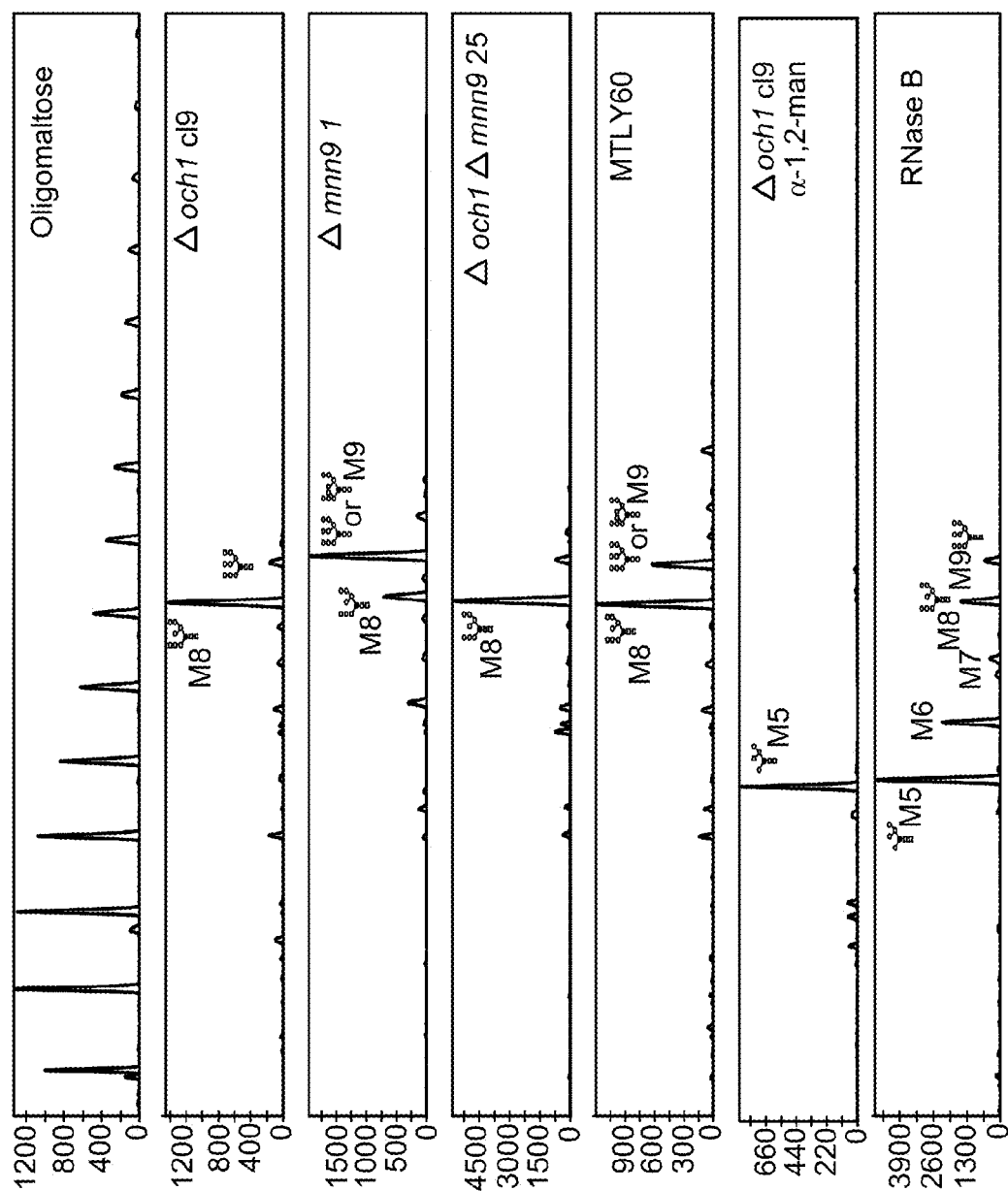
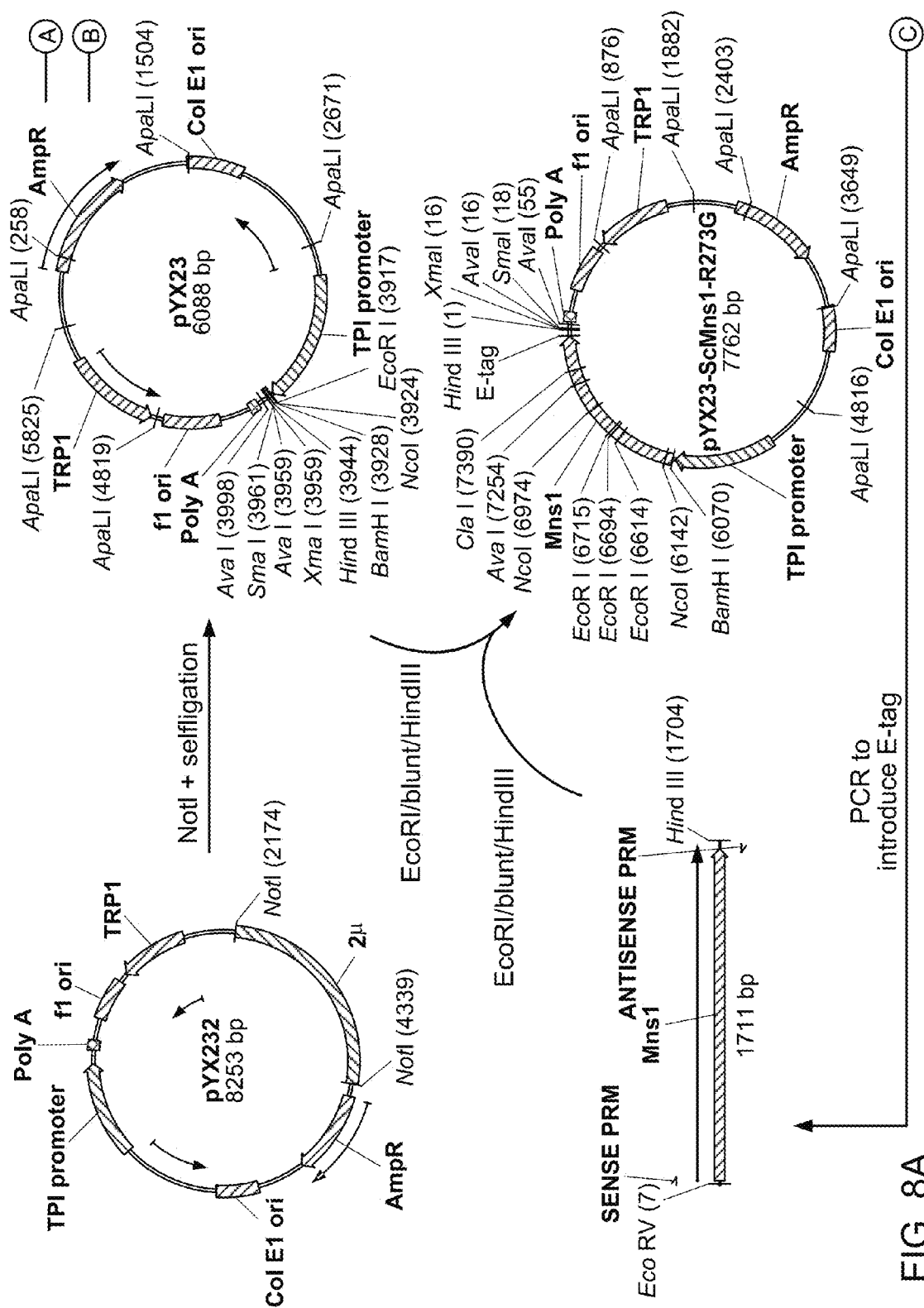
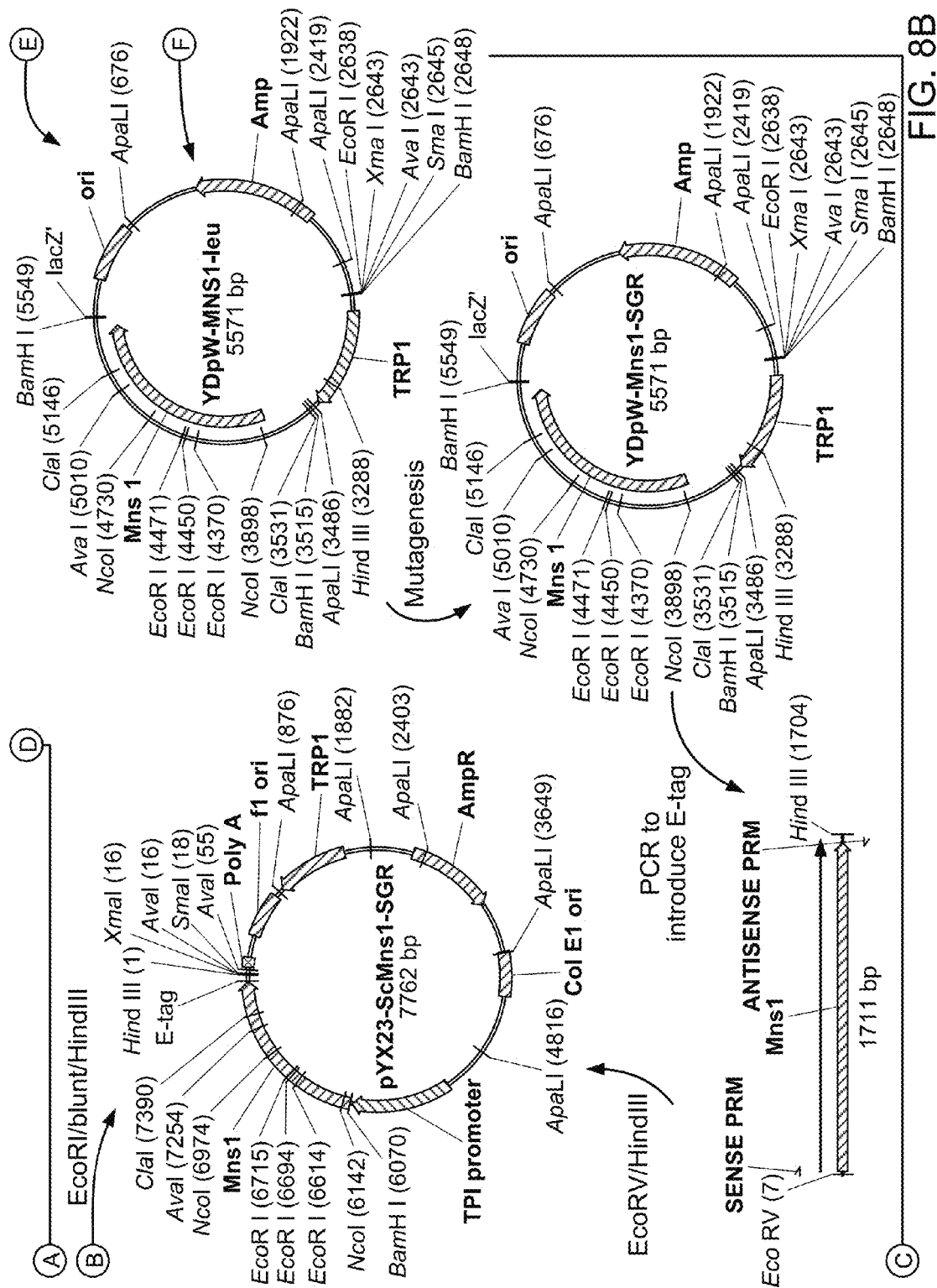


FIG. 7





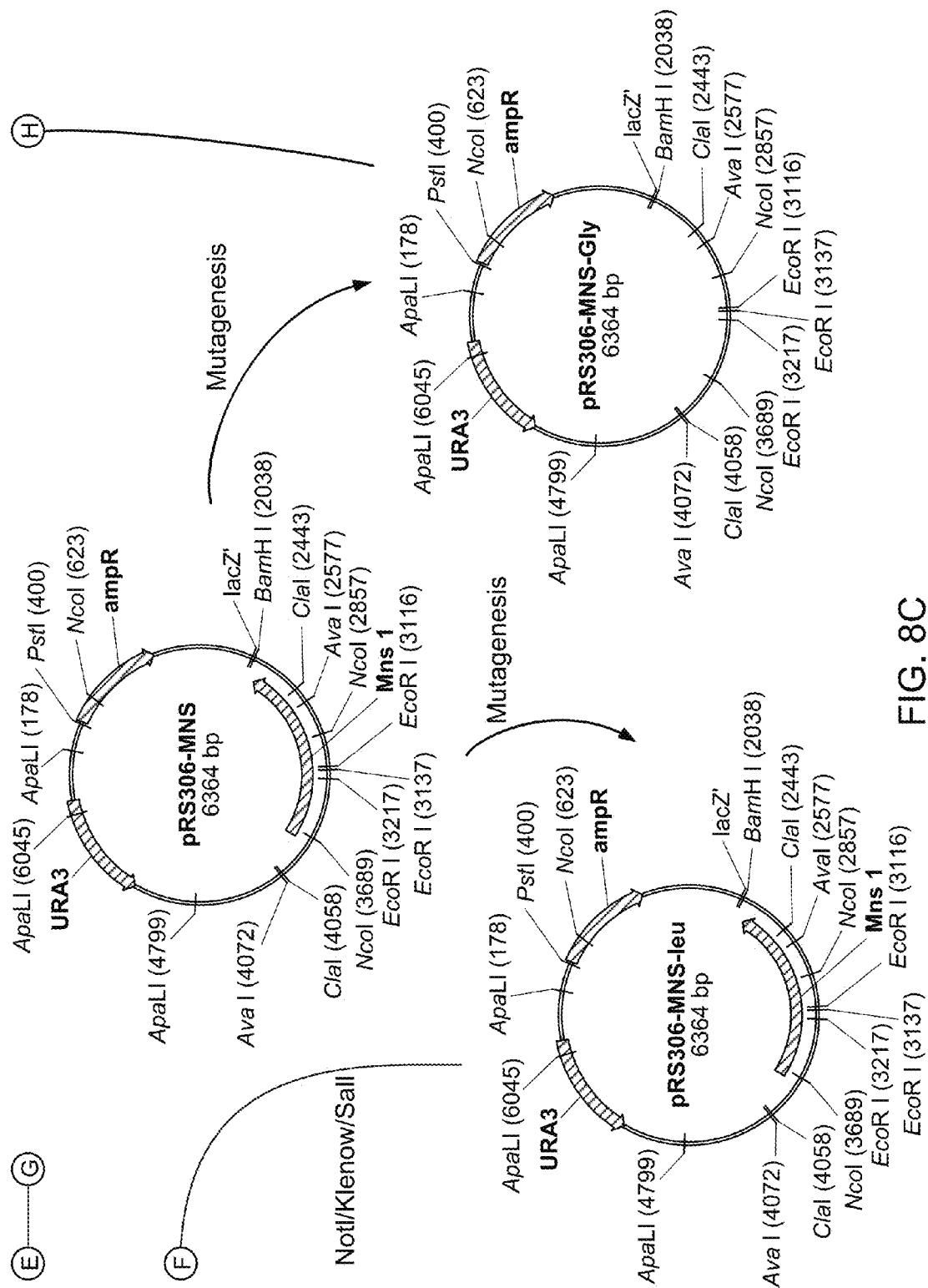


FIG. 8C

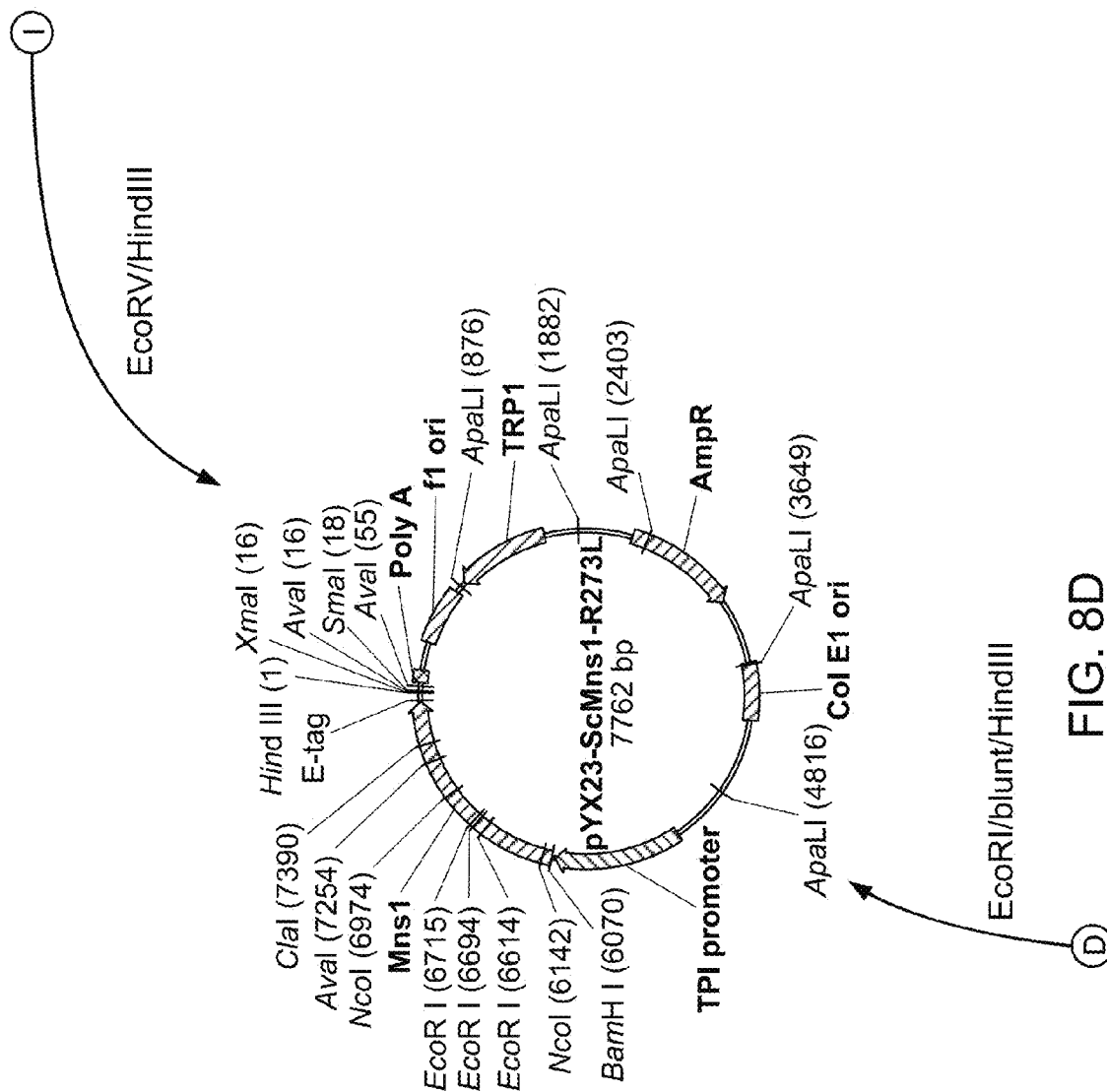
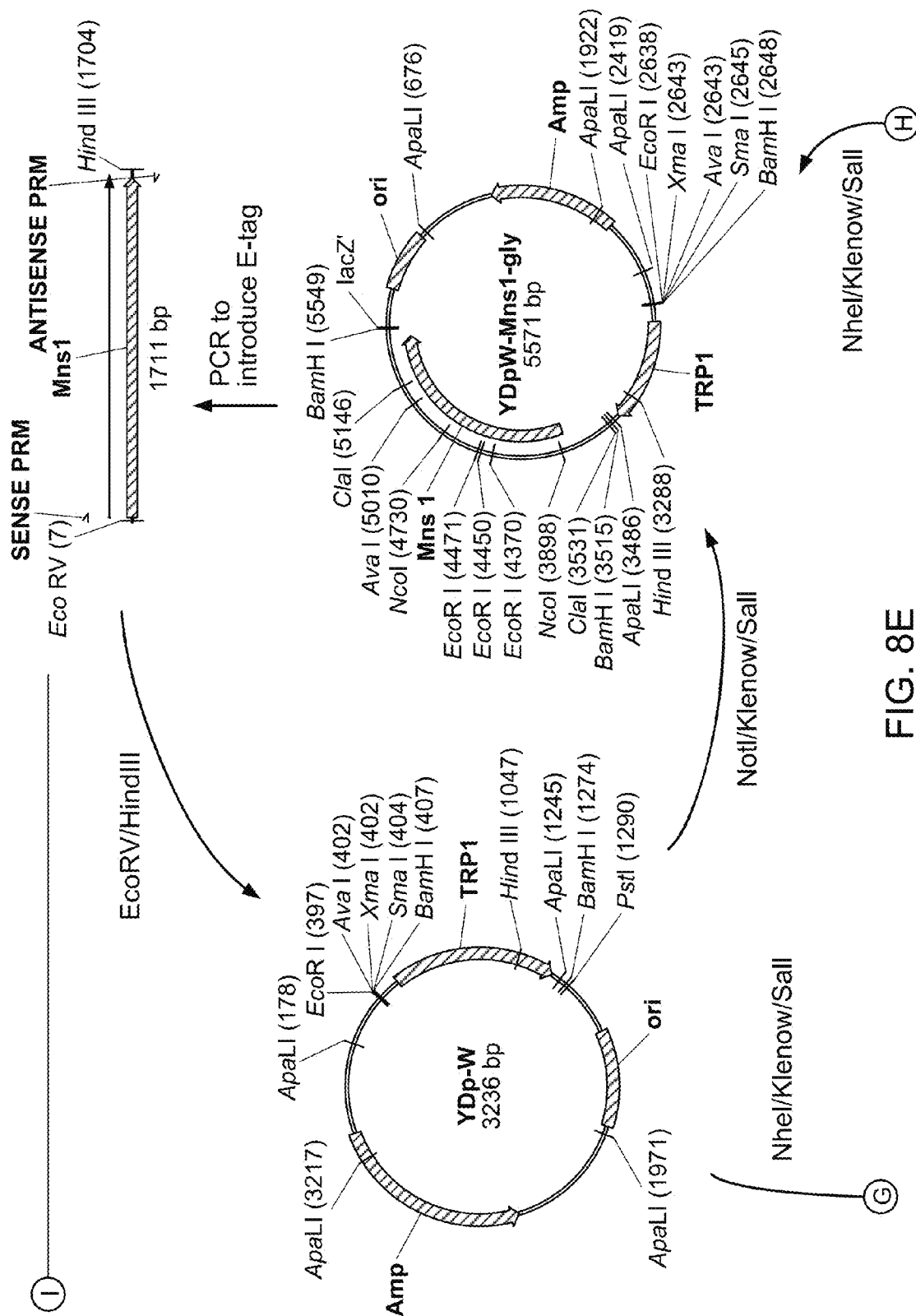


FIG. 8D



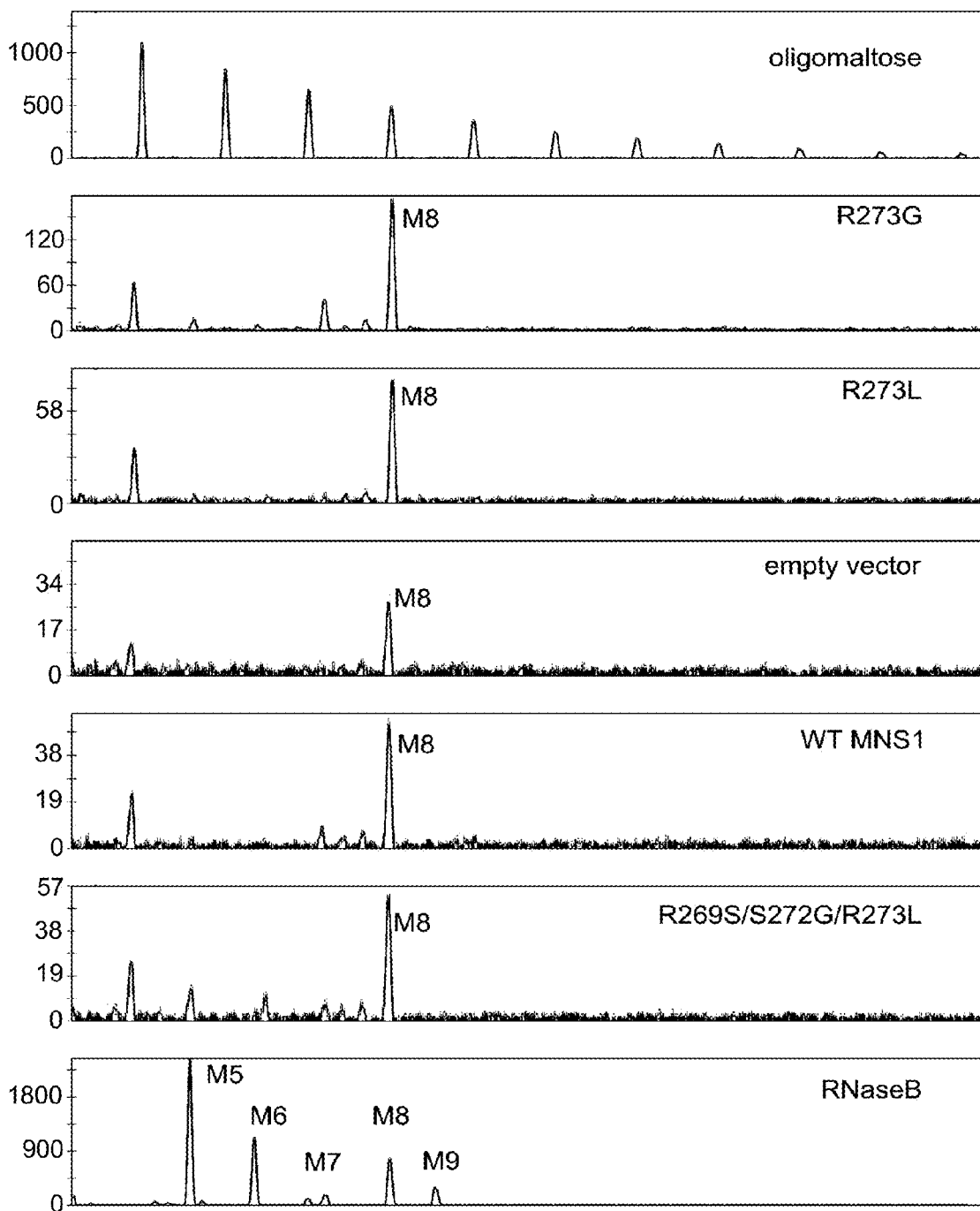


FIG. 9

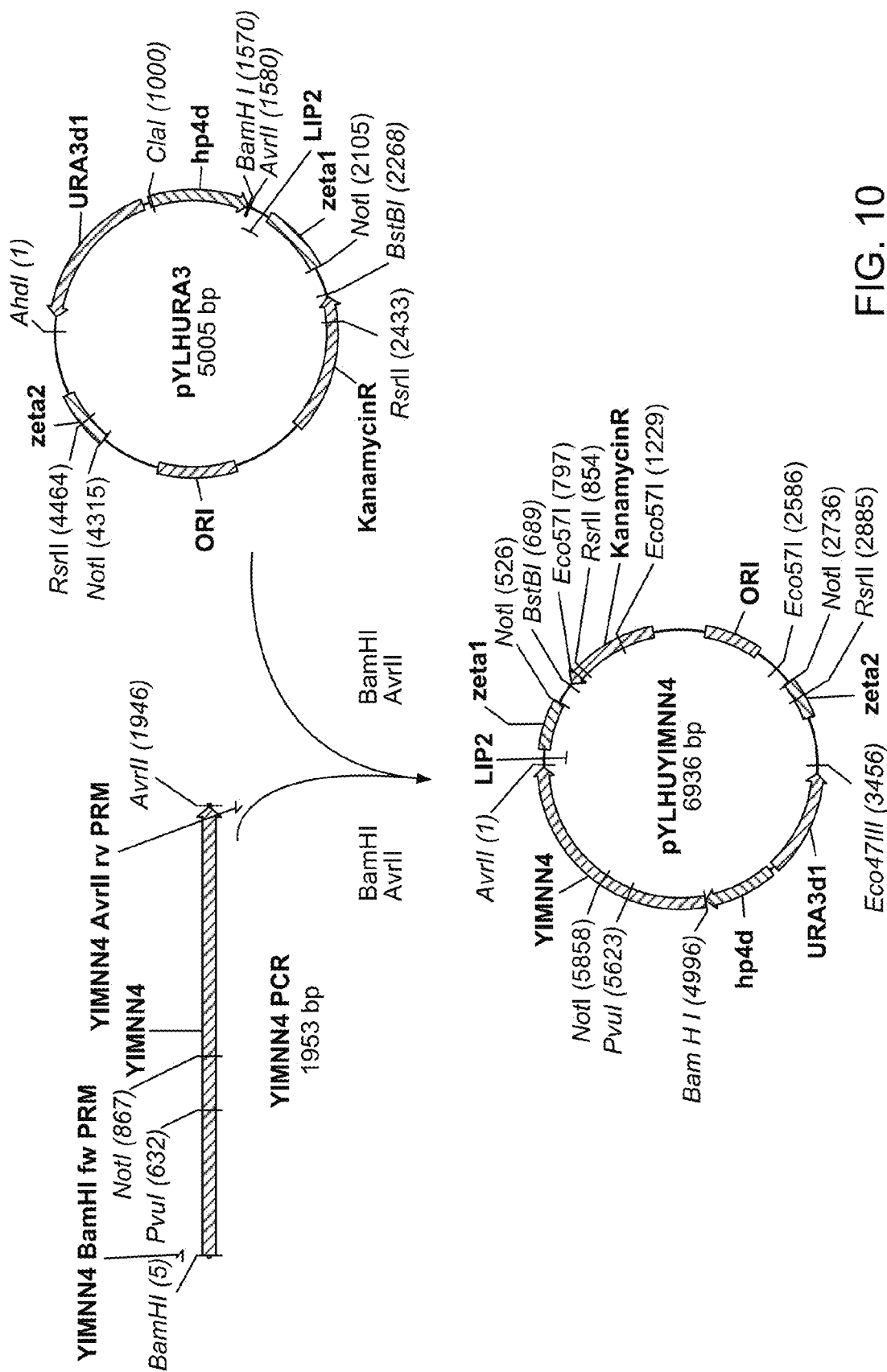


FIG. 10

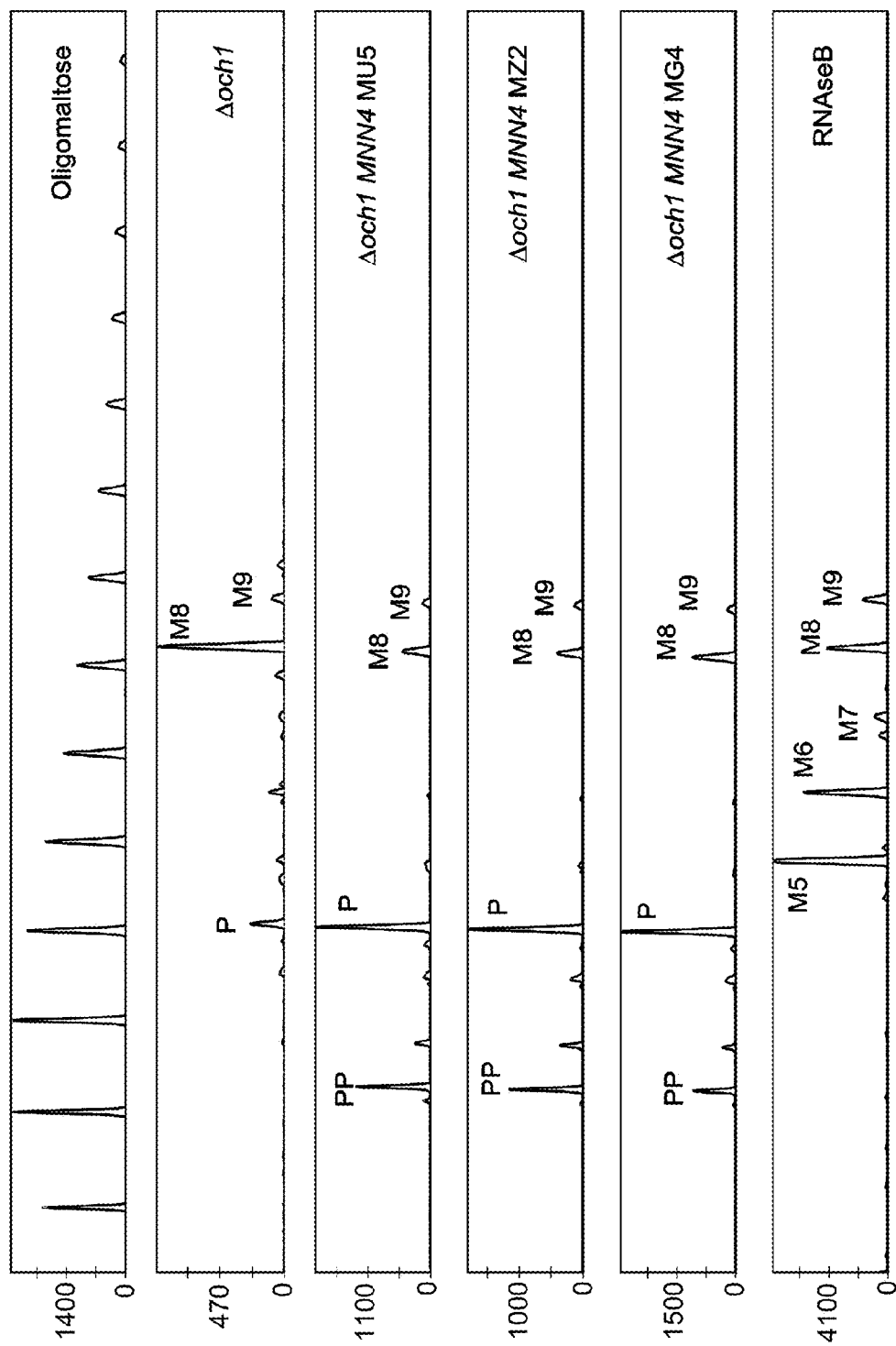


FIG. 11

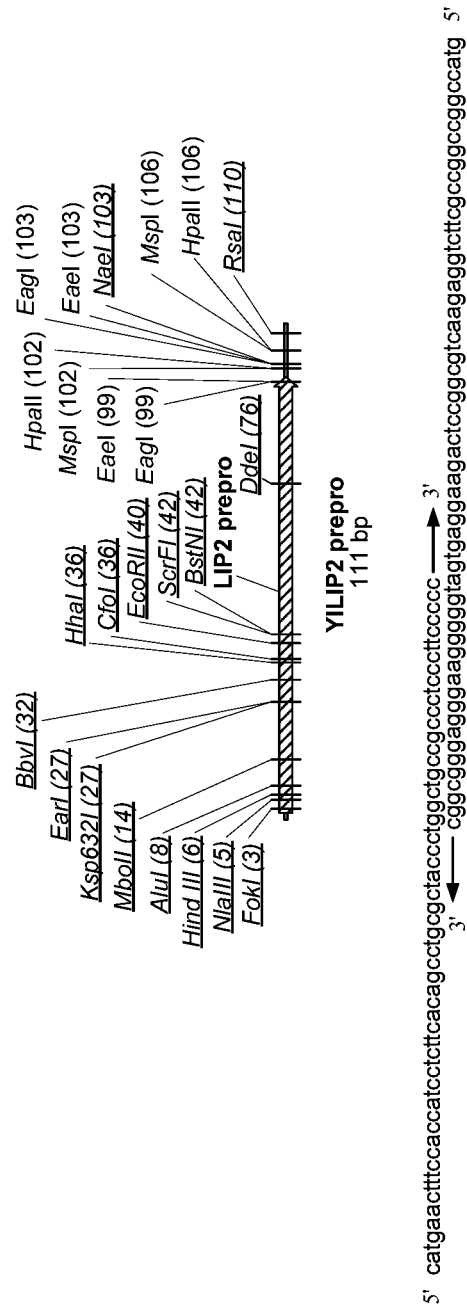


FIG. 12A

A

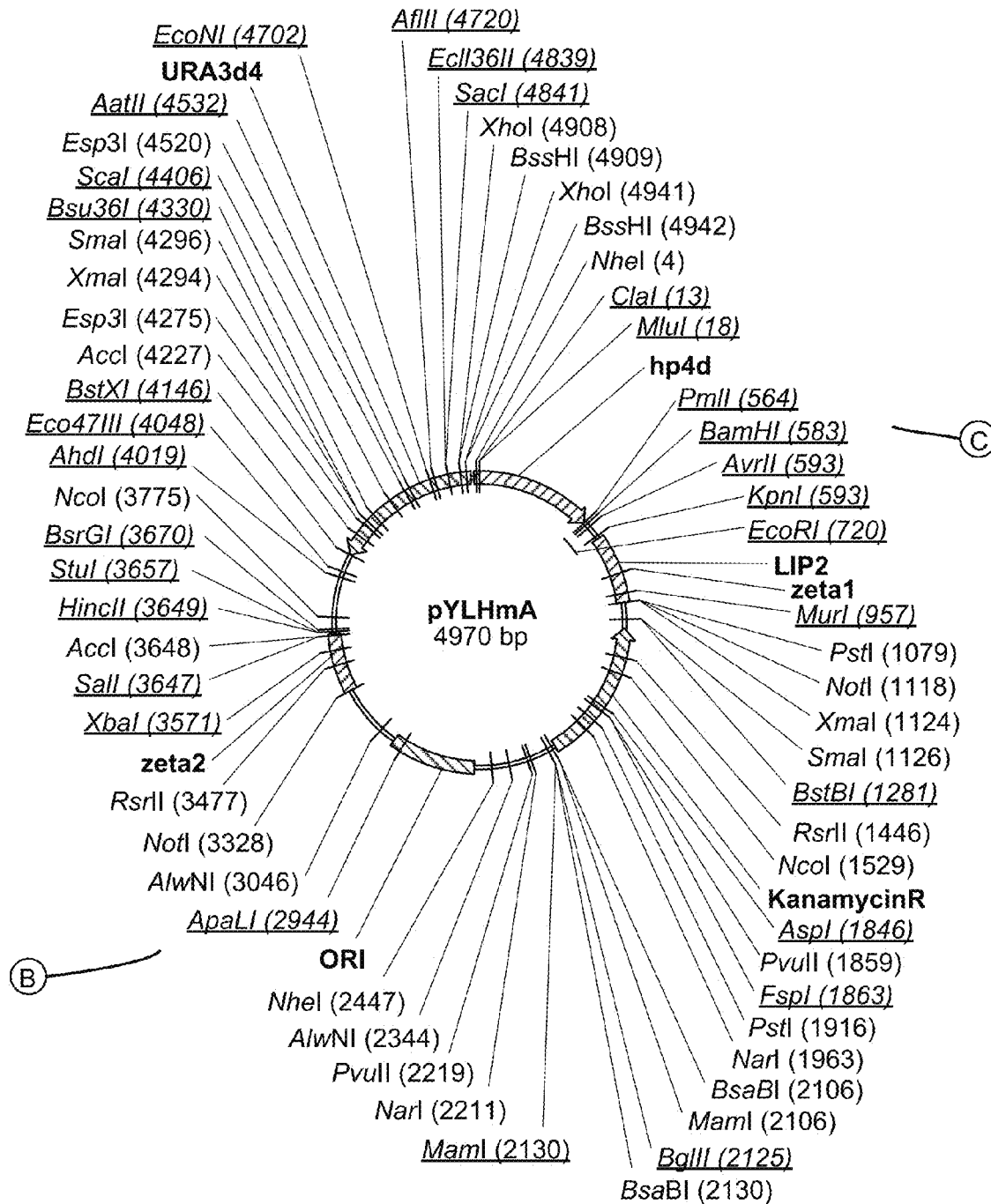


FIG. 12B

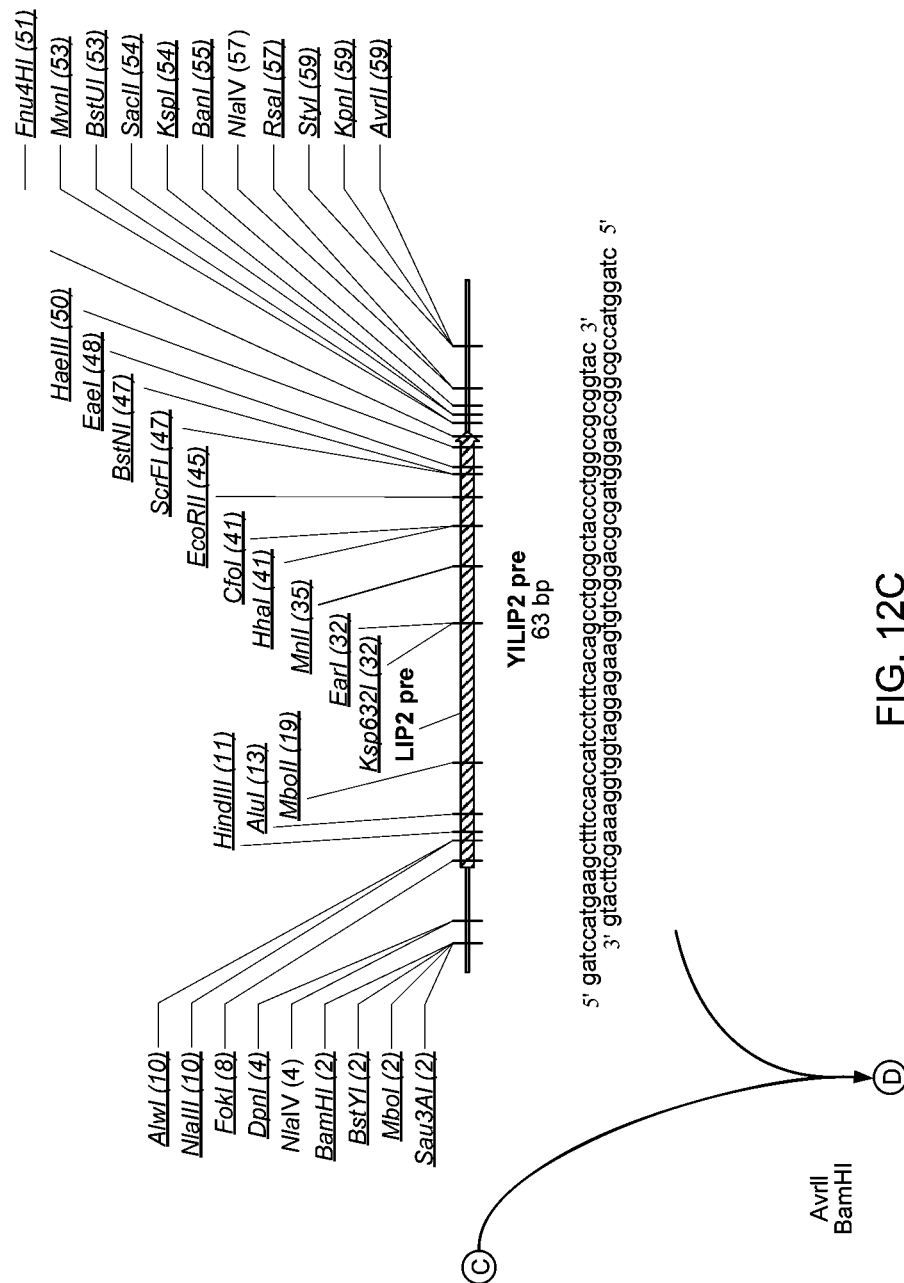


FIG. 12C

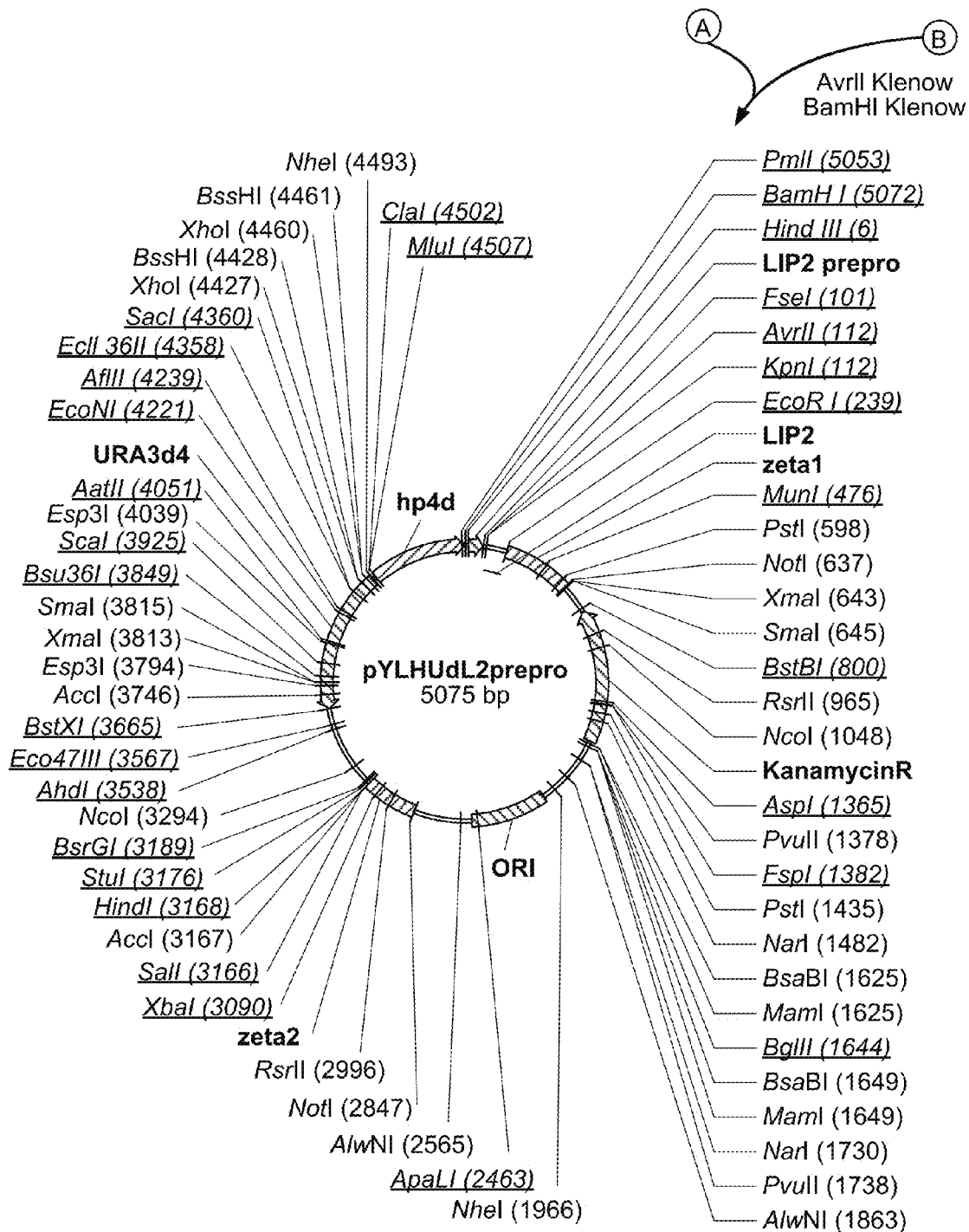


FIG. 12D

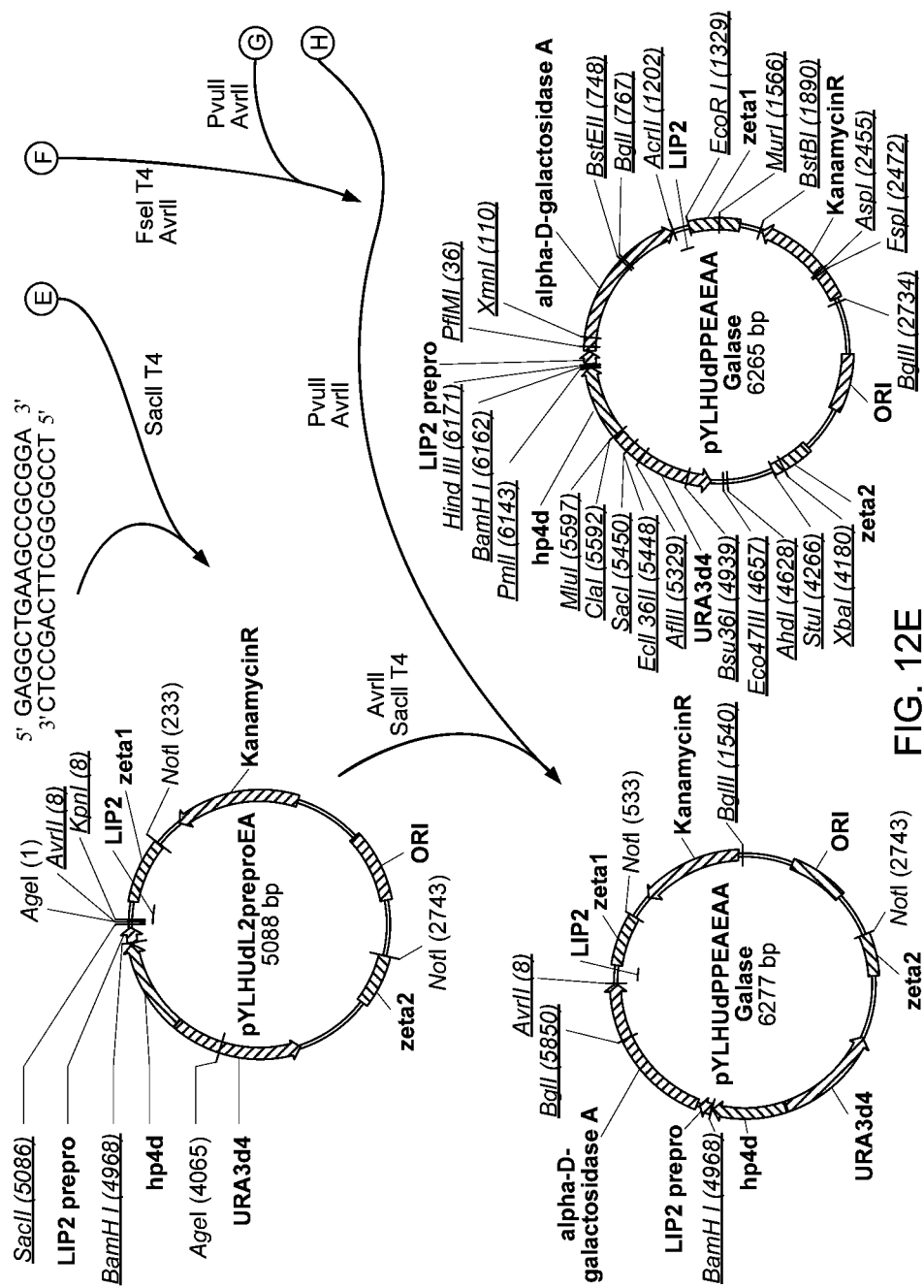


FIG. 12E

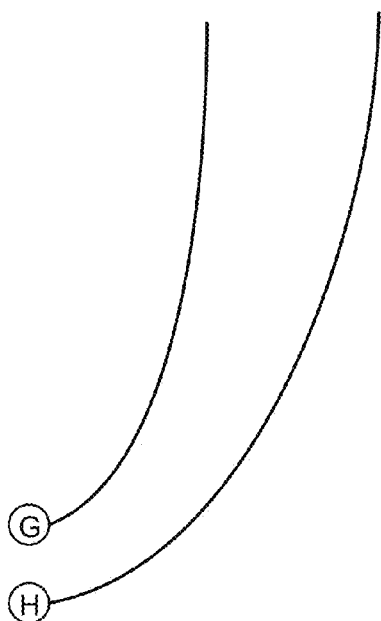
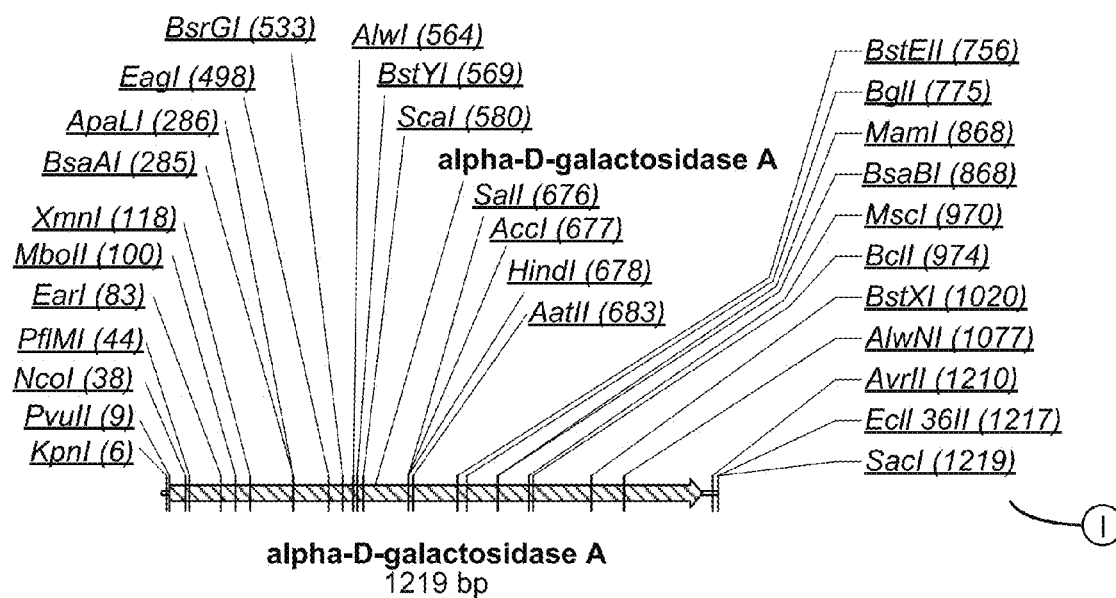
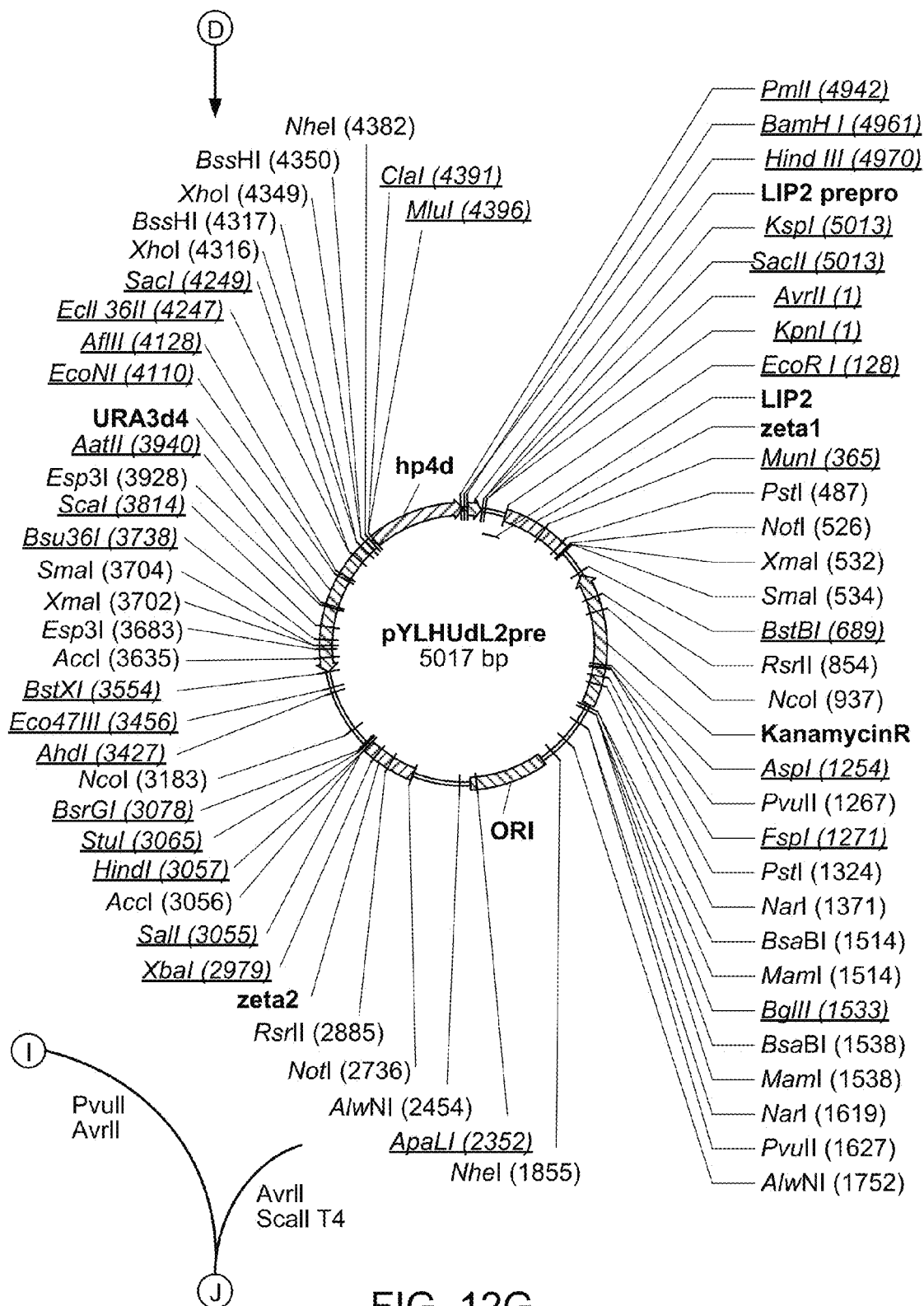


FIG. 12F



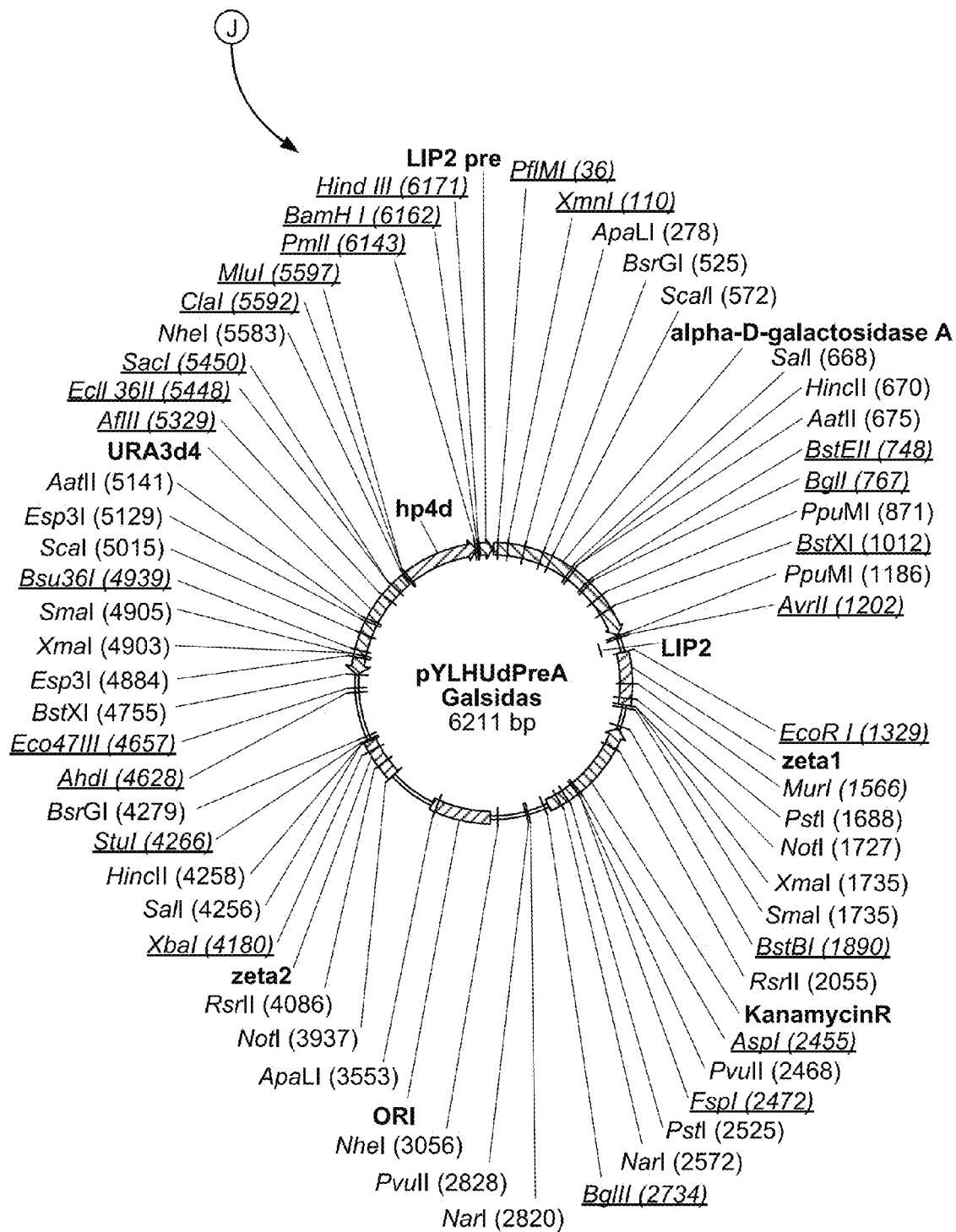


FIG. 12H

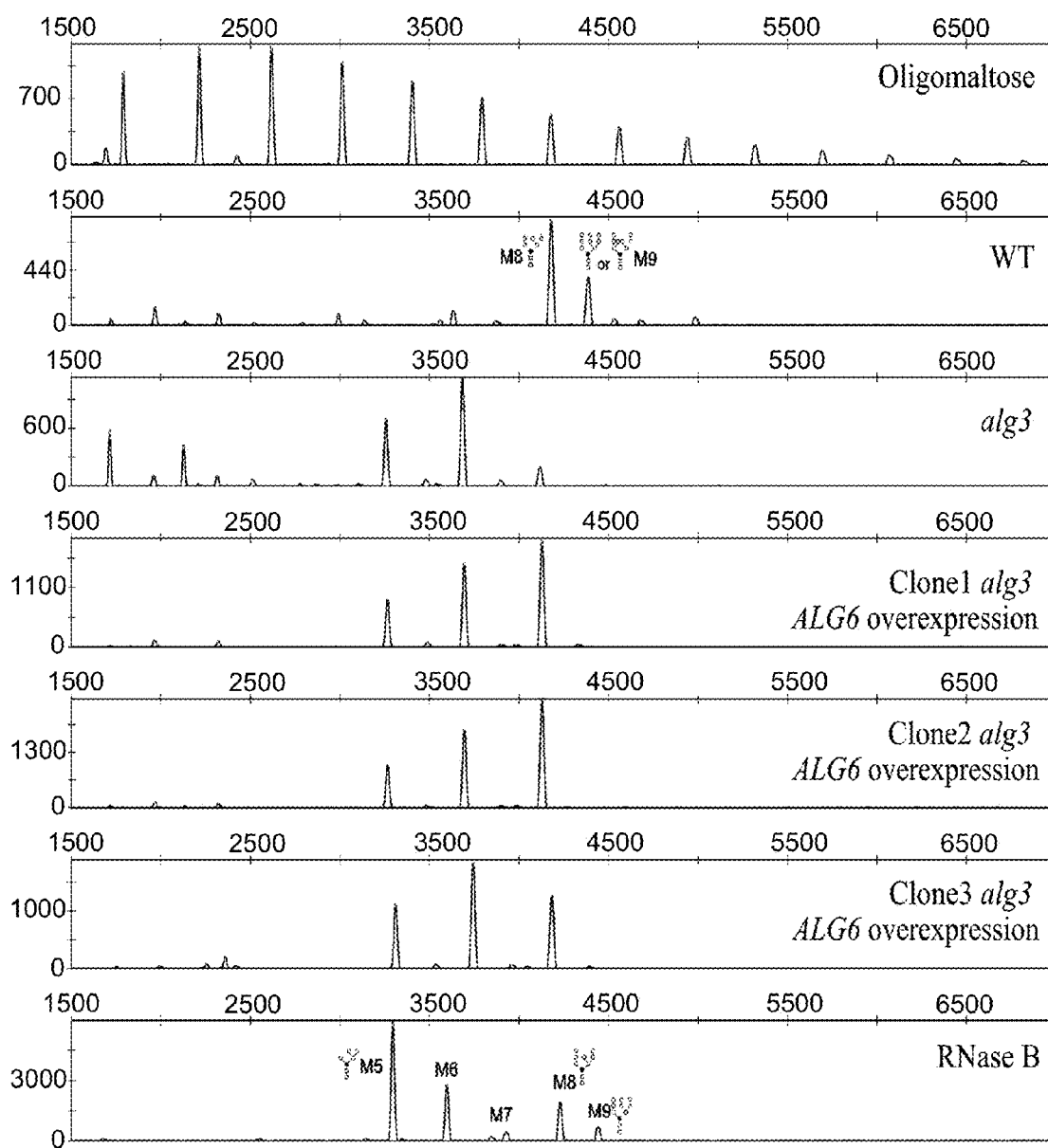


FIG. 13

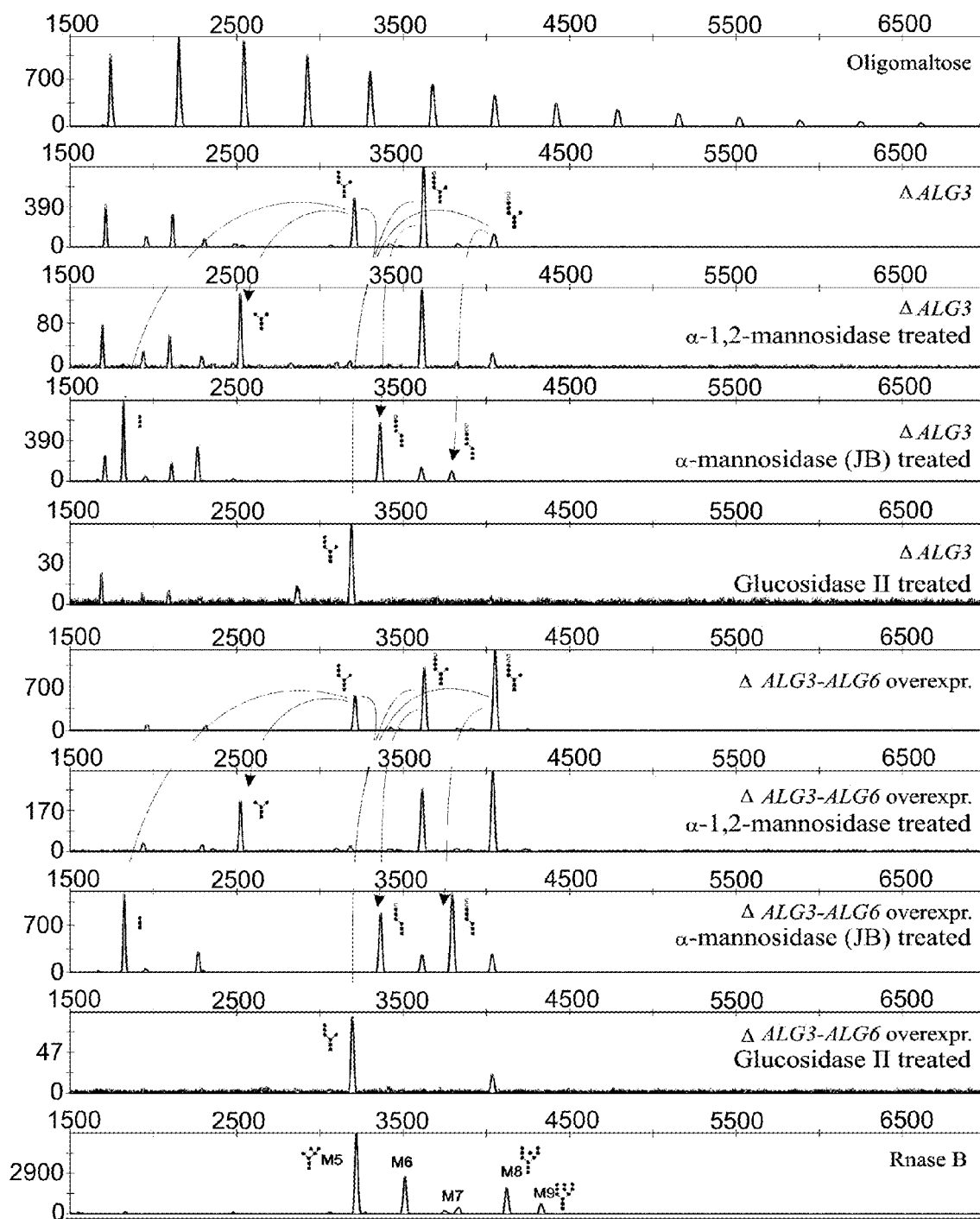


FIG. 14

FIG. 15

5' splice site

*P. pastoris* atccagcagtgatgacg

*S. cerevisiae* atccagccgtgattacg

\*\*\*\*\* \*\*\*\*\* \*\*\*

3' splice site

*P. pastoris* ggtctgcagcaccat

*S. cerevisiae* tgtccgaagcgcagt

\*\*\* \* \*\*\* \* \*

FIG. 16

NI.	1	atgcccgtagattcttctcataagacagctagcccacttccacctcgtaa	50
I.	1	atgcccgtagattcttctcataagacagctagcccacttccacctcgtaa	50
NI.	51	aagagcaaagacggaagaagaaaaggagcagcgctcgagtggaaacgtatcc	100
I.	51	aagagcaaagacggaagaagaaaaggagcagcgctcgagtggaaacgtatcc	100
NI.	101	tacgtaataggagagcggcccatgcttccagagagaagaaacgaagacac	150
I.	101	tacgtaataggagagcggcccatgcttccagagagaagaaacgaagacac	150
NI.	151	gttgaatttctggaaaaccacgctcgacctggaatctgcacttcaaga	200
I.	151	gttgaatttctggaaaaccacgctcgacctggaatctgcacttcaaga	200
NI.	201	atcagccaaagccactaacaagttgaaagaaatacaagatatcattgttt	250
I.	201	atcagccaaagccactaacaagttgaaagaaatacaagatatcattgttt	250
NI.	251	caaggttggaagccttaggtggtaccgtctcagatttggatttaacagtt	300
I.	251	caaggttggaagccttaggtggtaccgtctcagatttggatttaacagtt	300
NI.	301	cgggaagtcgattttcccaaattcttctgatttggaaacctatgtctgatct	350
I.	301	cgggaagtcgattttcccaaattcttctgatttggaaacctatgtctgatct	350
NI.	351	ctcaacttcttcgaaaatcggagaaagcatctacatccactcgcagatctt	400
I.	351	ctcaacttcttcgaaaatcggagaaagcatctacatccactcgcagatctt	400
NI.	401	tgactgaggatctggacgaagatgacgtcgctgaatatgacgacgaagaa	450
I.	401	tgactgaggatctggacgaagatgacgtcgctgaatatgacgacgaagaa	450
NI.	451	gaggacgaagagttacccaggaaaatgaaagtcttaaacgacaaaaacaa	500
I.	451	gaggacgaagagttacccaggaaaatgaaagtcttaaacgacaaaaacaa	500
NI.	501	gagcacatctatcaagcaggagaagttgaatgaacttccatctcctttgt	550
I.	501	gagcacatctatcaagcaggagaagttgaatgaacttccatctcctttgt	550
NI.	551	catccgatttttccagacgtagatgaagaaaagtcaactctcacacattta	600
I.	551	catccgatttttccagacgtagatgaagaaaagtcaactctcacacattta	600
NI.	601	aagttgcaacagcaacaacaacaaccagtagacaattatgttttctactcc	650
I.	601	aagttgcaacagcaacaacaacaaccagtagacaattatgttttctactcc	650

FIG. 17A

NI.	651	tttgagtcttccggaggattcagttgattttattaacccaggtaacttaa	700
I.	651	tttgagtcttccggaggattcagttgattttattaacccaggtaacttaa	700
NI.	701	aaatagagtccgatgagaacttcttggttgagttcaaatactttacaaata	750
I.	701	aaatagagtccgatgagaacttcttggttgagttcaaatactttacaaata	750
NI.	751	aaacacgaaaatgacaccgactacattactacagctccatcaggttccat	800
I.	751	aaacacgaaaatgacaccgactacattactacagctccatcaggttccat	800
NI.	801	caatgatttttttaattcttattgacattagcgagtcgaatcggttgcac	850
I.	801	caatgatttttttaattcttattgacattagcgagtcgaatcggttgcac	850
NI.	851	atccagcagtgatgacggattcattctttacacattacagcaggctccatc	900
I.	851	atccag-----	855
NI.	901	ggctttttctcttttgattgggggggggaaagtctctgtagcagggaggcg	950
I.	856	-----	855
NI.	951	cagttcagttggcacatatcagttgacatgcatagcgatcaggtgatgc	1000
I.	856	-----	855
NI.	1001	tcttgtaactggtatgccaagtcacacggttttcggcgctacctactactc	1050
I.	856	-----	855
NI.	1051	tactttaactcttgaattgagatgaaacaatcgcccttggtcgagacgcg	1100
I.	856	-----	855
NI.	1101	tagttaatttaagatgaatcaatttgcaacaaaagaacaaaacgaaaaaga	1150
I.	856	-----	855
NI.	1151	taacagcaaaacttaagtttcgtggtctgcagcaccatttaccgctaatagc	1200
I.	856	-----cagcaccatttaccgctaatagc	878
NI.	1201	atttgatttaaatgactttgtattcttccaggaatag	1237
I.	879	atttgatttaaatgactttgtattcttccaggaatag	915

FIG. 17B

P.pastoris	1	APFTANA <b>FDLNDF</b> VFFQE
18		
		..... : :    .....
S.cerevisiae	1	EAQSG <b>LN</b> S <b>FELNDF</b> FITS
18		

FIG. 18

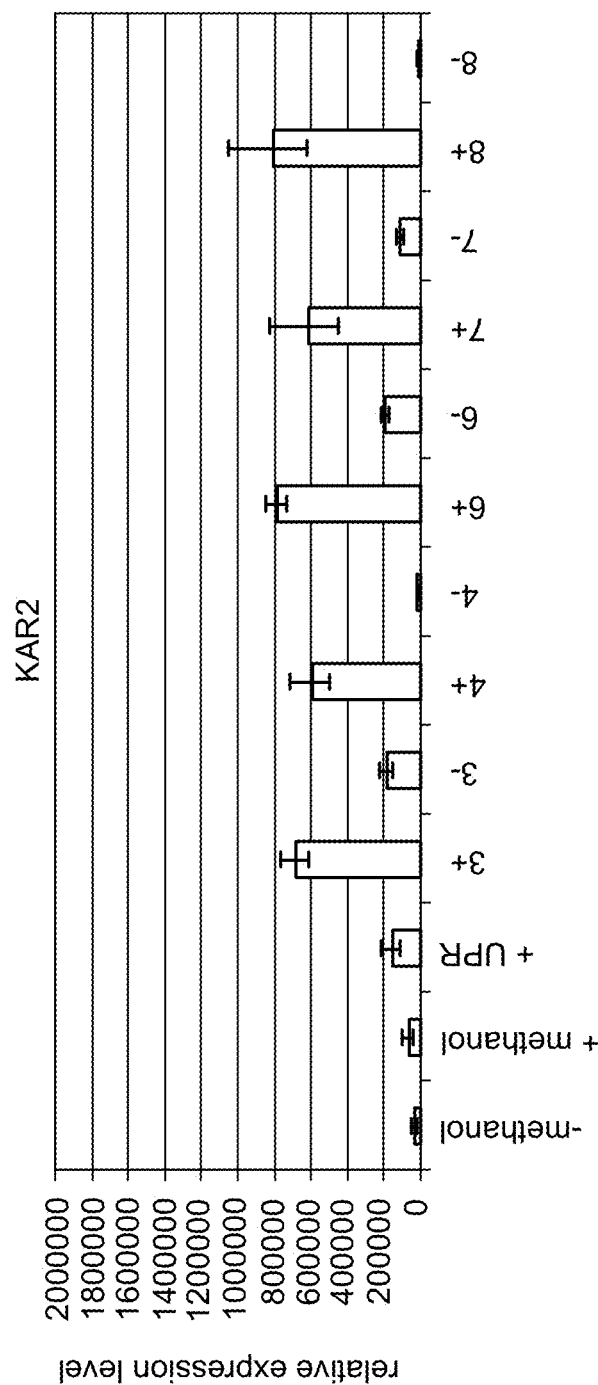
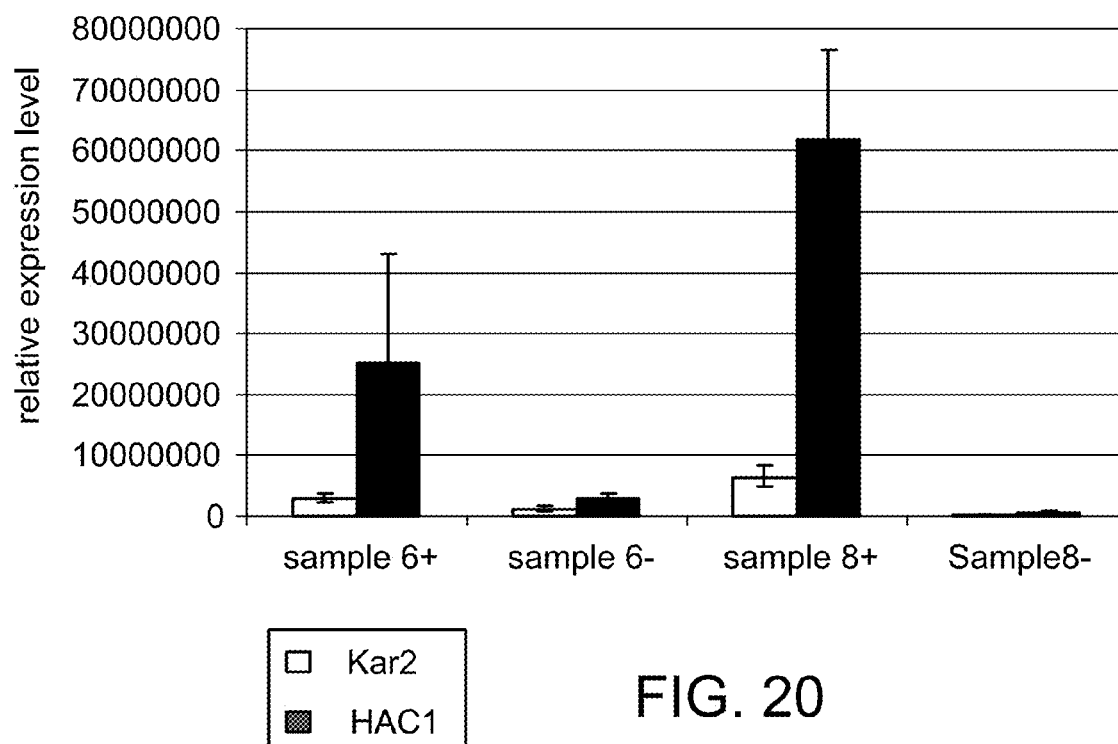


FIG. 19



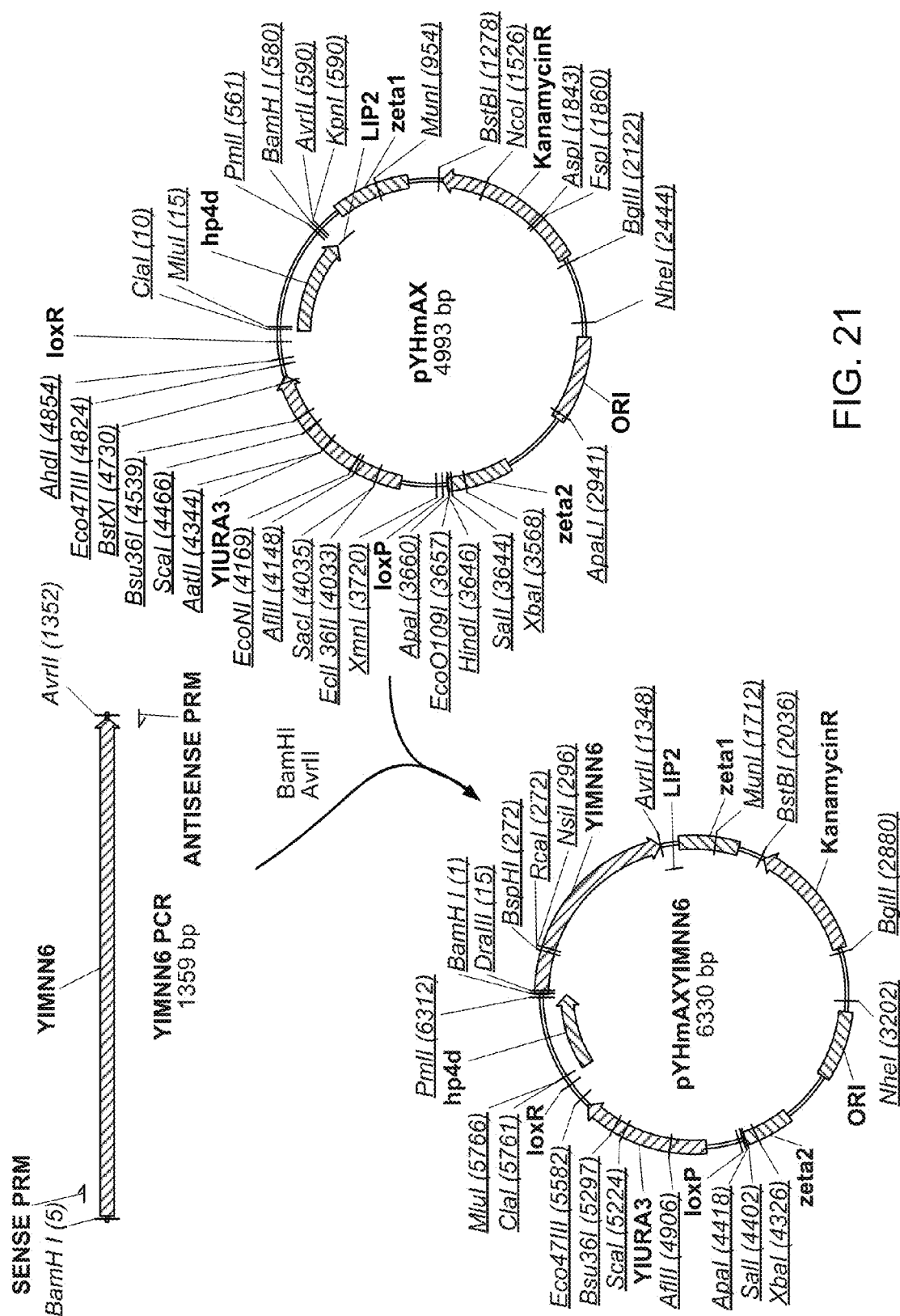


FIG. 21

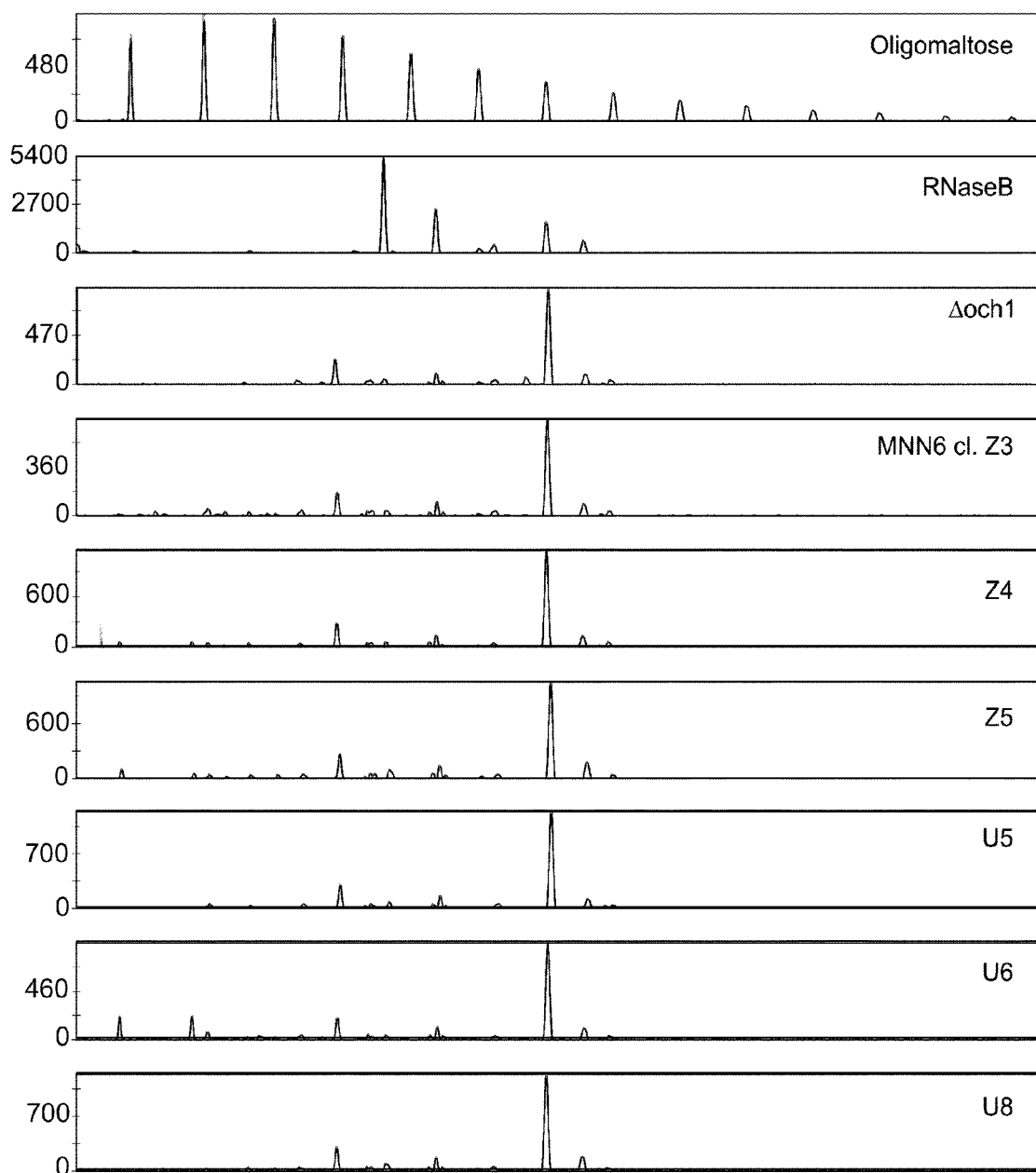


FIG. 22

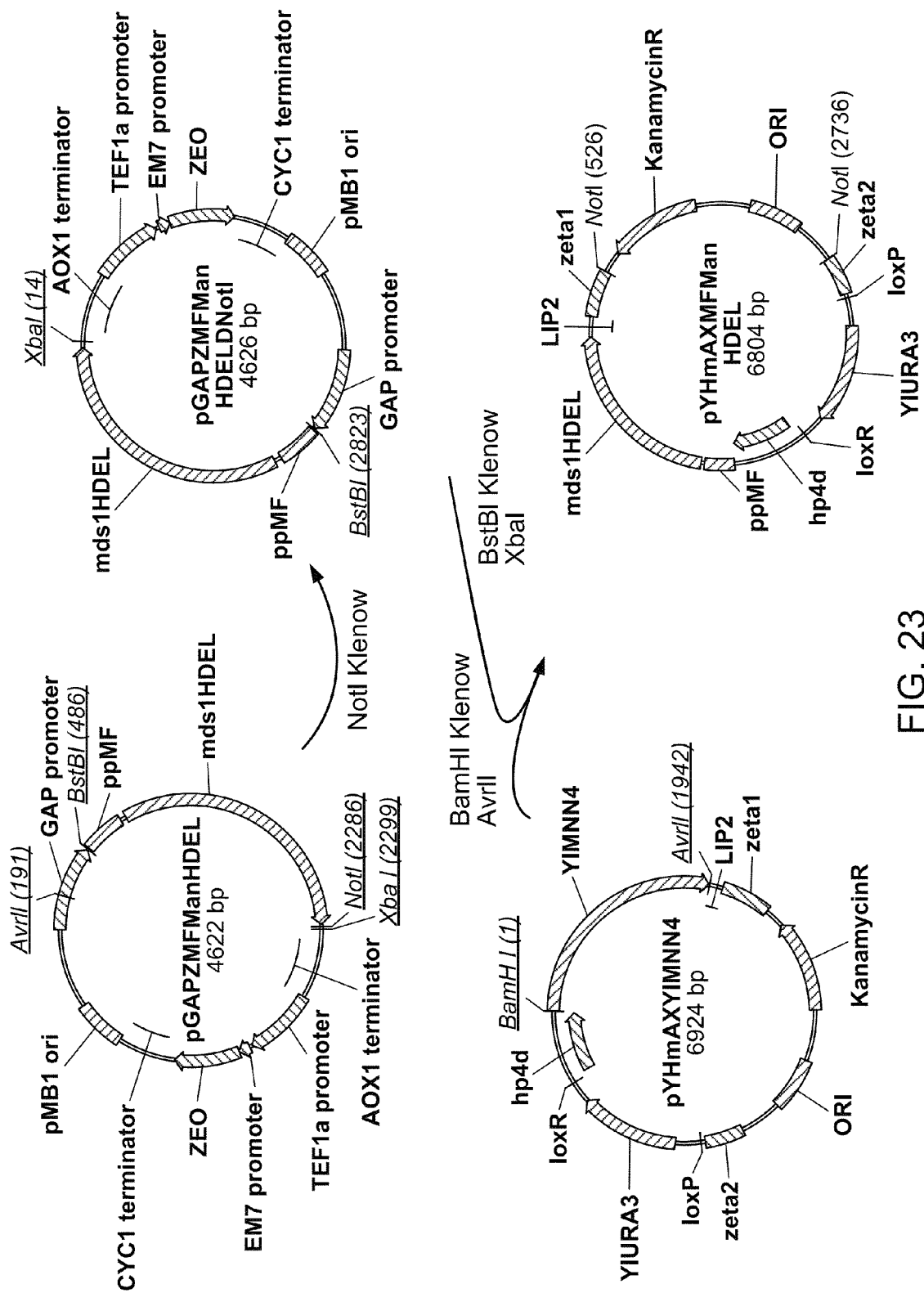


FIG. 23

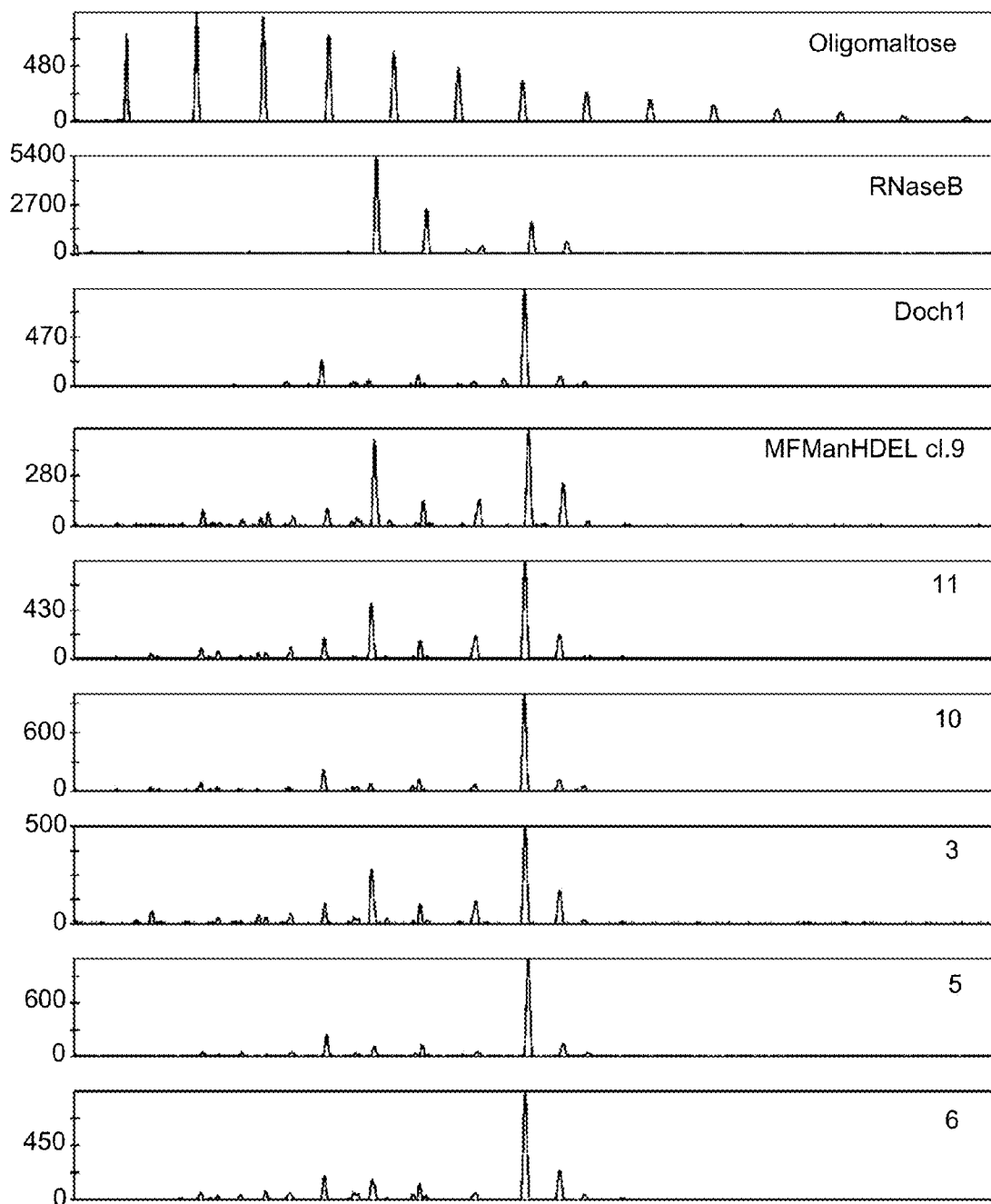
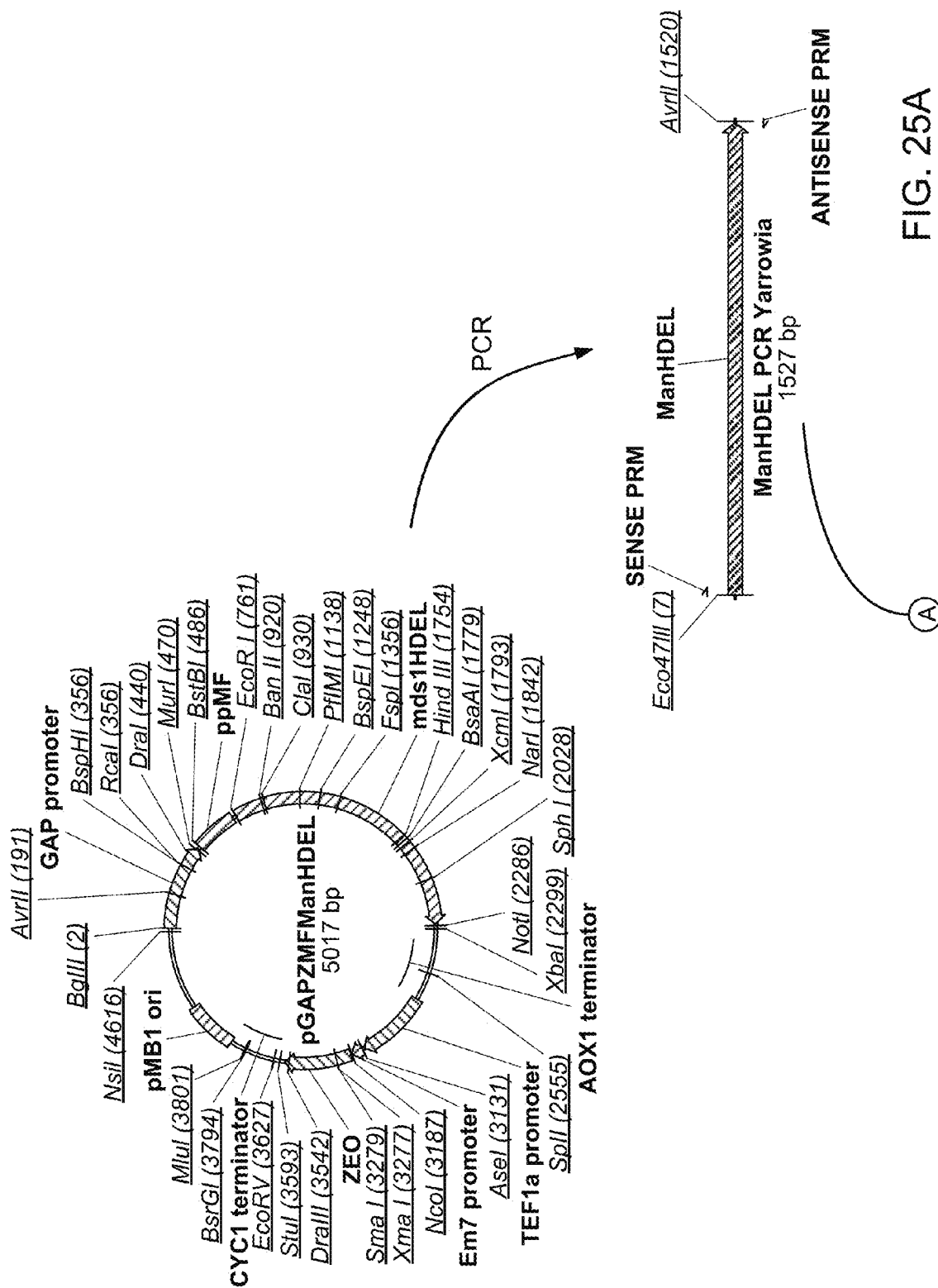


FIG. 24



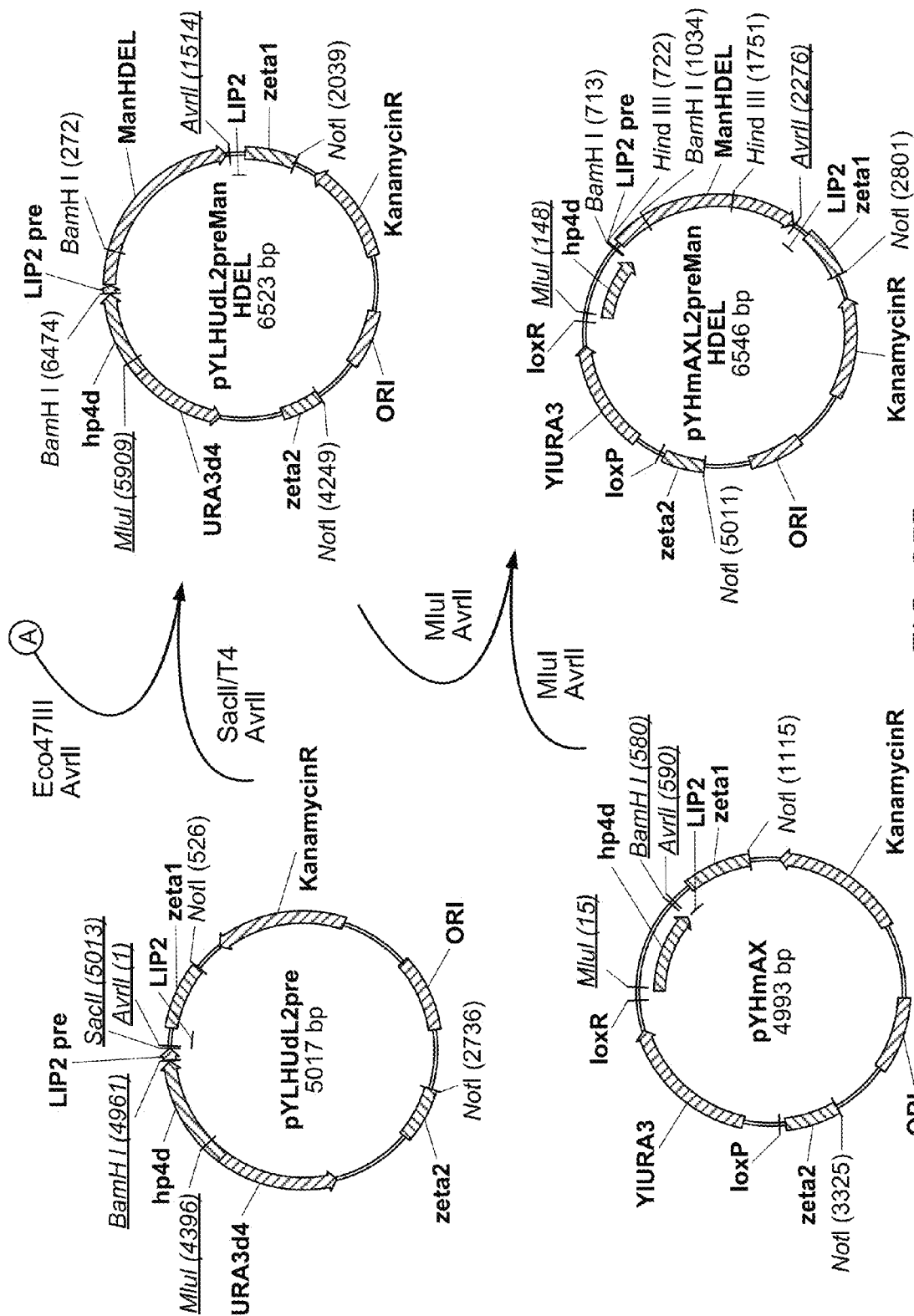


FIG. 25B

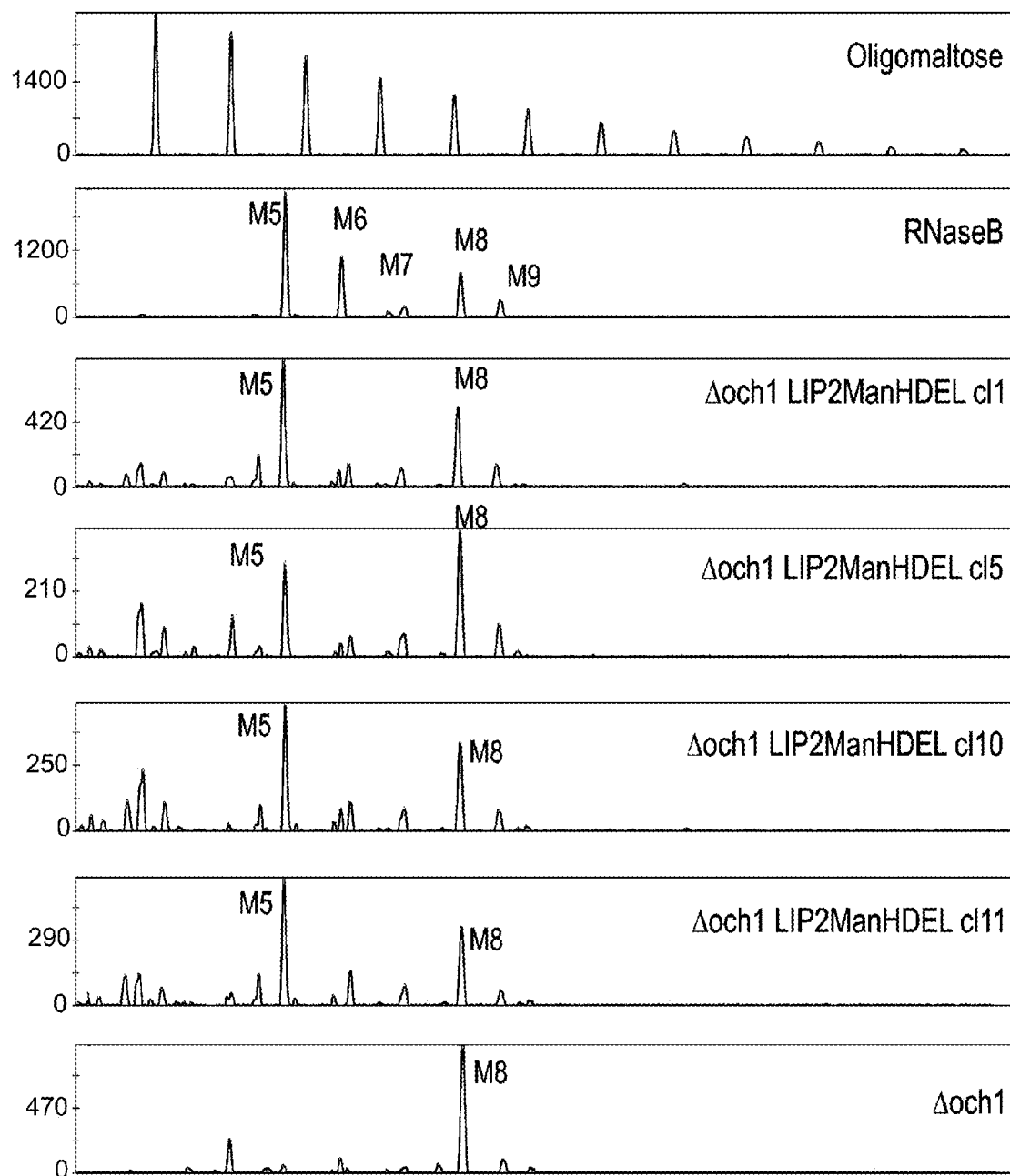


FIG. 26

MSIKREESFTPTPEDLGSPLTADSPGSPESGDKRKKDLTLP  
LPAGALPPRKRAKTENEKEQRRIERIMRNRQAAHASREKKRRH  
LEDLEKKCSELSSSENNDLHHQVTESKKTNMHLMEQHYSLVAK  
LQQLSSLVNMAKSSGALAGVDVPDMSDVSMAPKLEMPAAPS  
QPMGLASAPTLFNHDNETVVPDSPIVKTEEV DSTNFLHTESSS  
PPELAESTGSGSPSSTLSCDETDYLVDRARHPAASAQDFIFIKD  
EPVDDELGLHGLSDDFTLFEDNKQPAQHDFIADLAHYESSVSN  
LFGGLE

FIG. 27A

MPVDSSHKTASPLPPRKRAKTEEEKEQRRVERILRNRRAA  
HASREKKRRHVFELENHVVDLESALQESAKATNKLKEIQDIIV  
SRLEALGGTVSDLDLTVPEVDFPKSSDLEPMSDLSTSSKSEKAS  
TSTRRLTEDLDEDDVAEYDDEEEDEELPRKMKVLNDKNKSTS  
IKQEKLNELPSPLSSDFSDVDEEKSTLTHLKLQQQQQQPVDNY  
VSTPLSLPEDSVDFINPGNLKIESDENFLLSSNTLQIKHENDTD  
YITTAPSGSINDFFNSYDISESNRLHHPAAPFTANAFDLNDFVF  
FQE

FIG. 27B

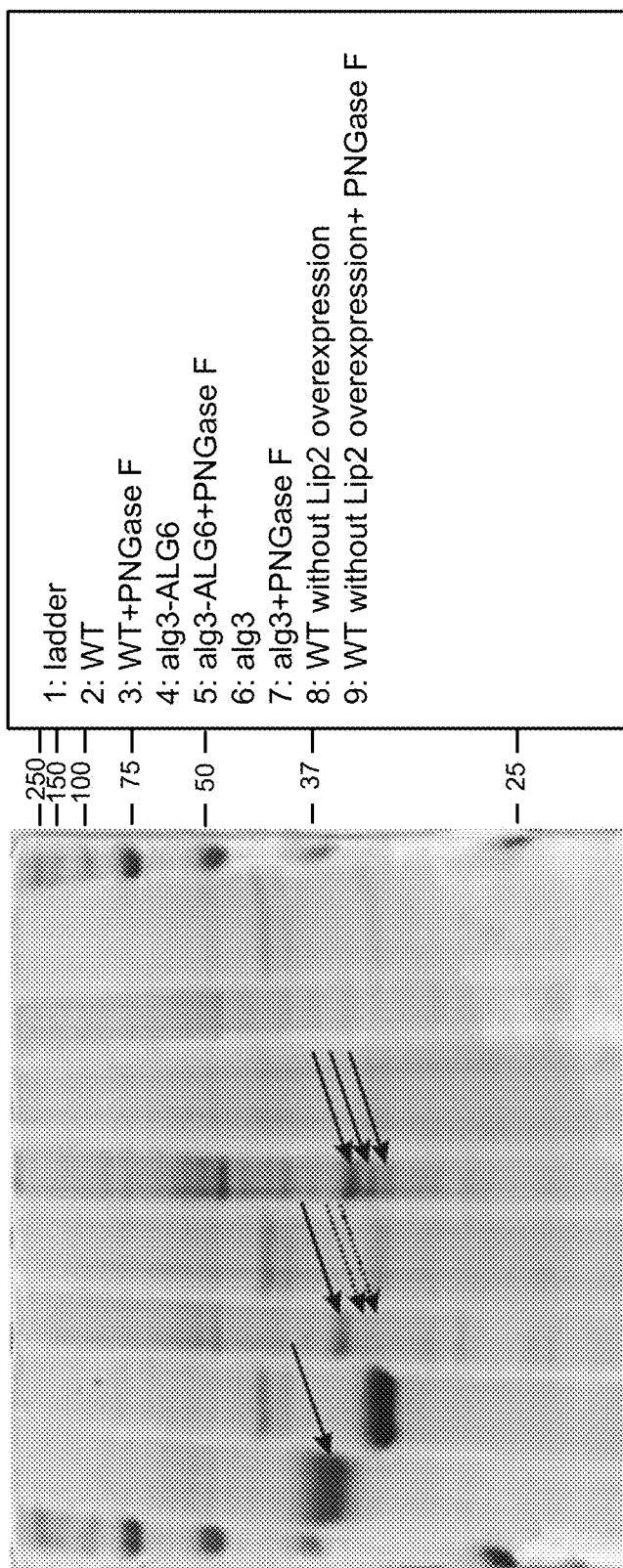


FIG. 28

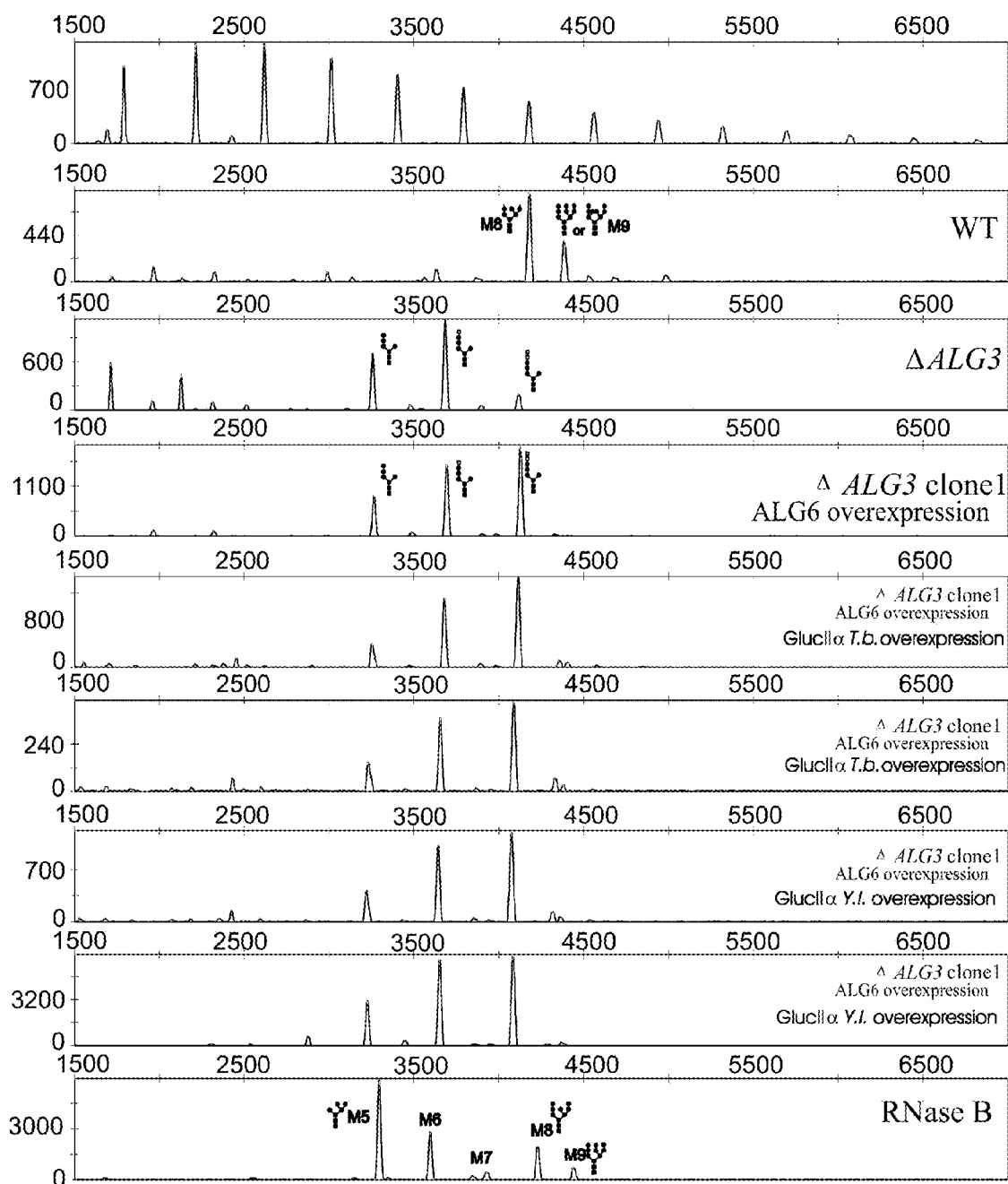


FIG. 29

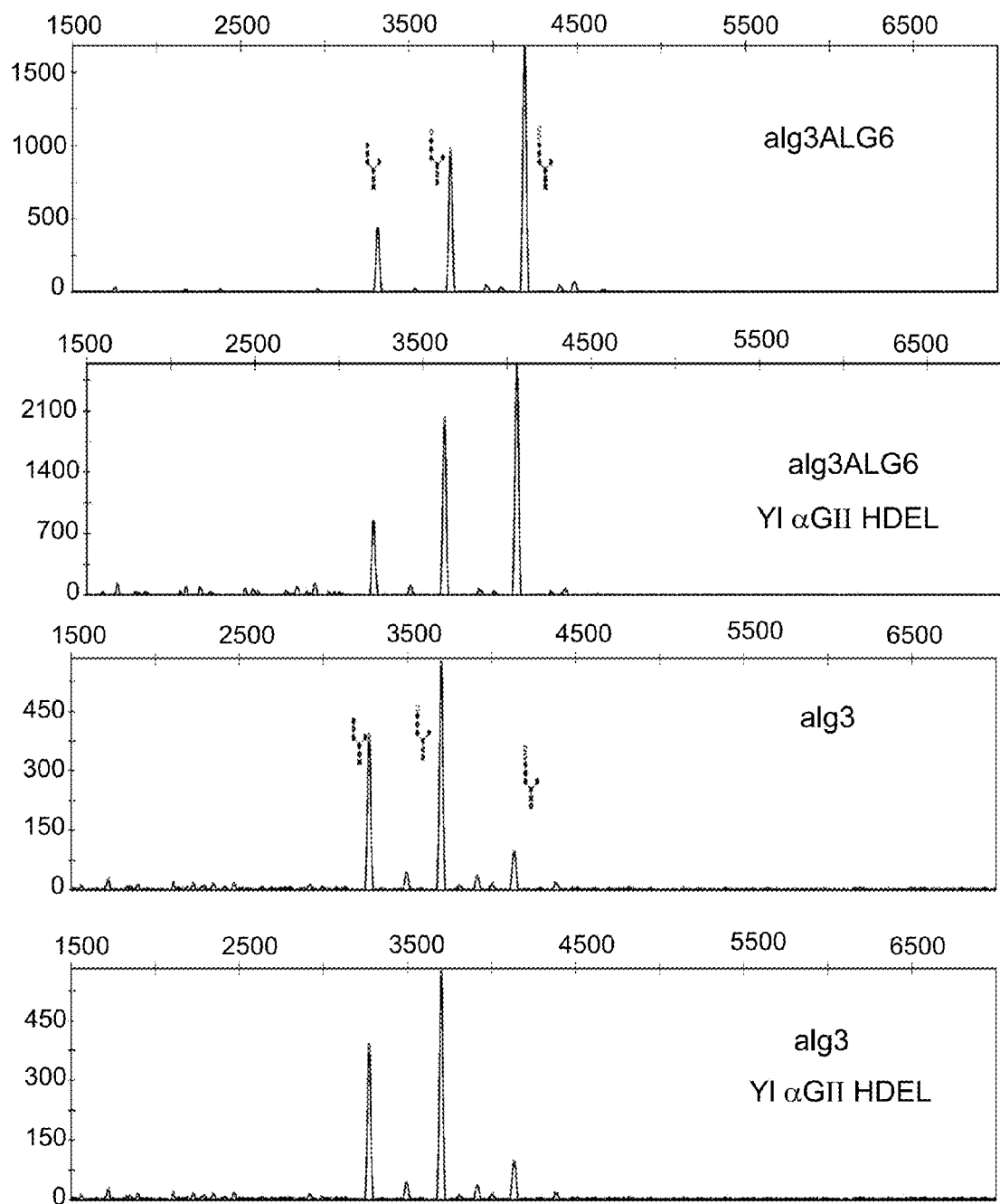


FIG. 30

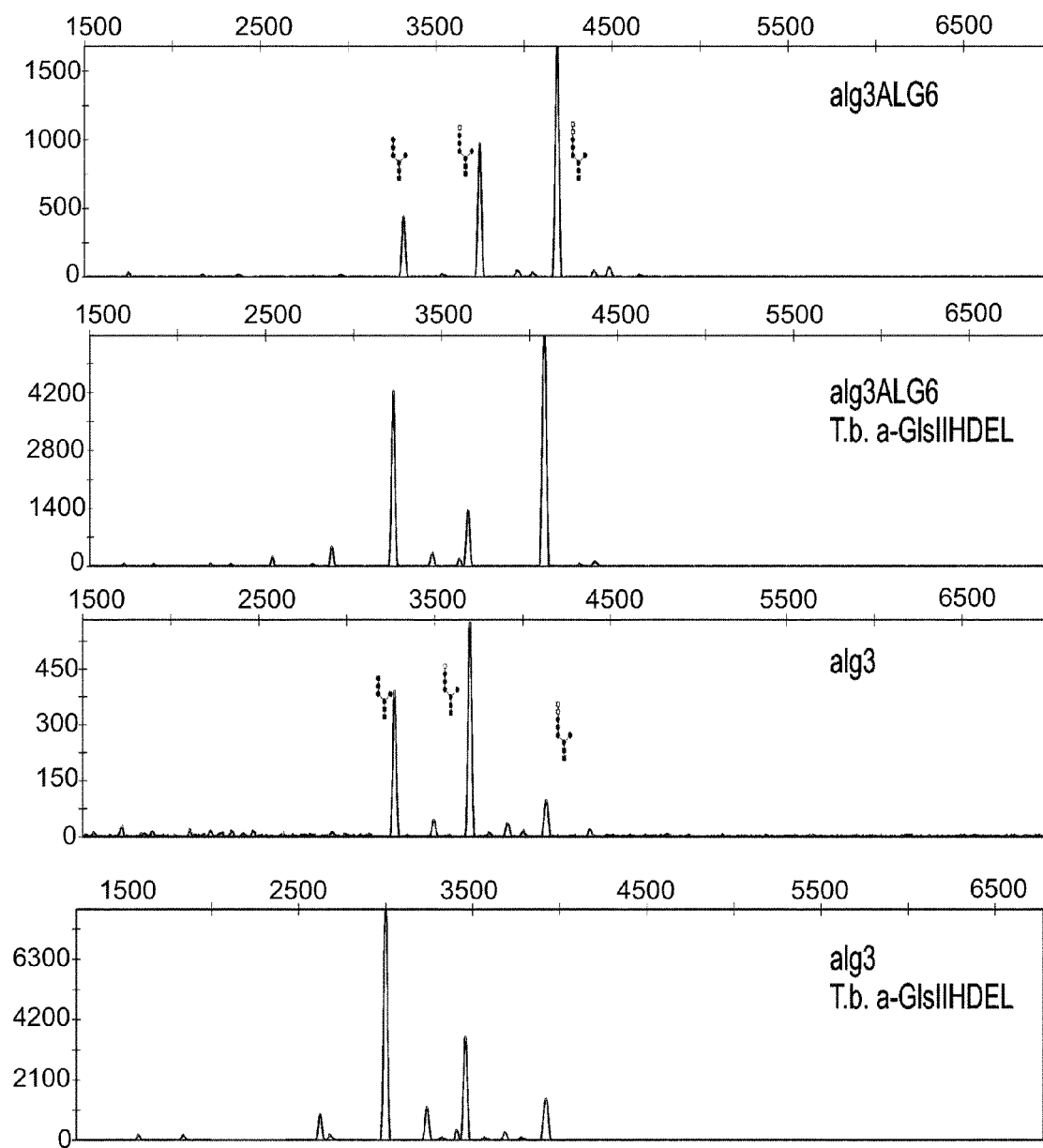


FIG. 31

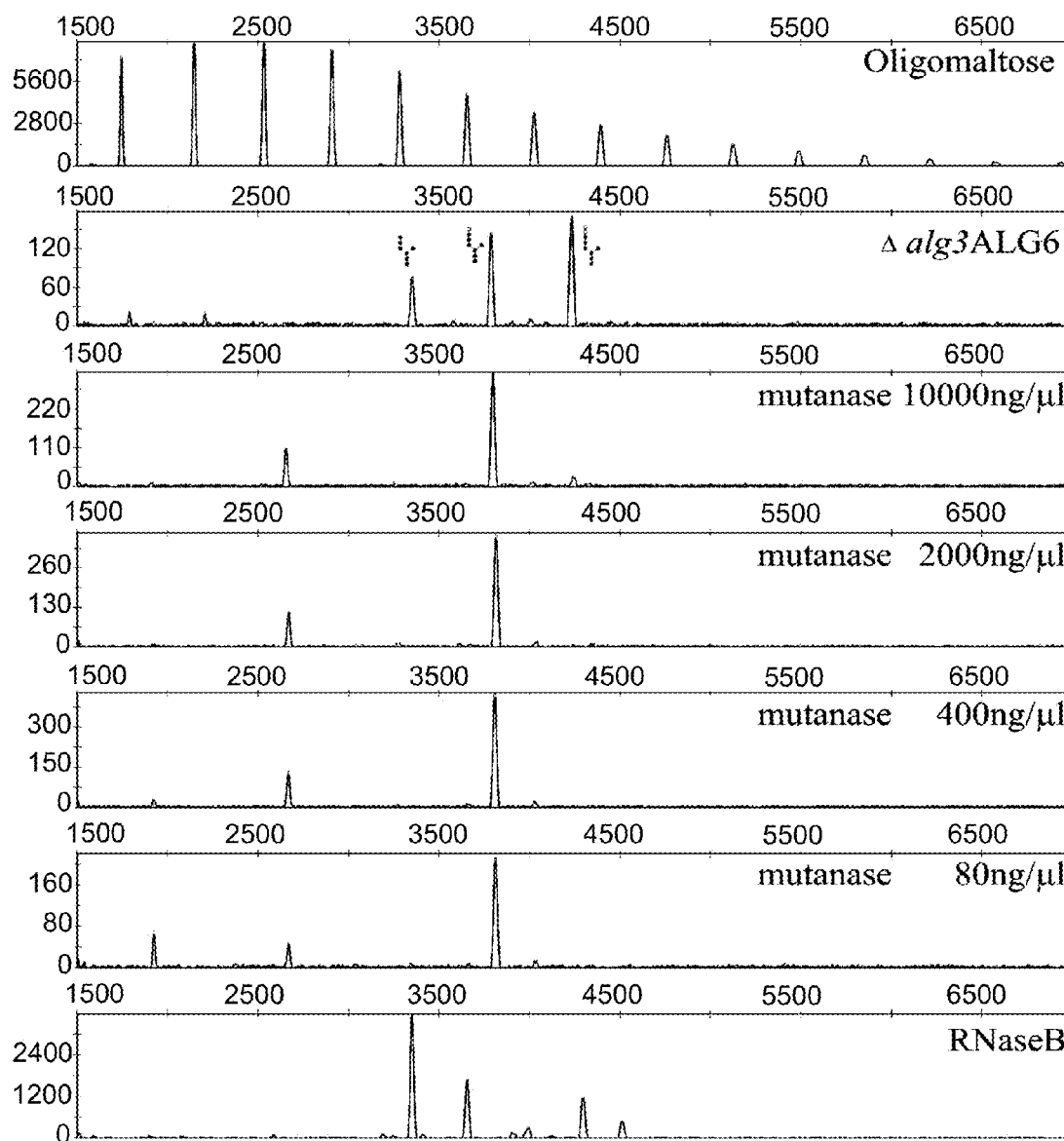


FIG. 32

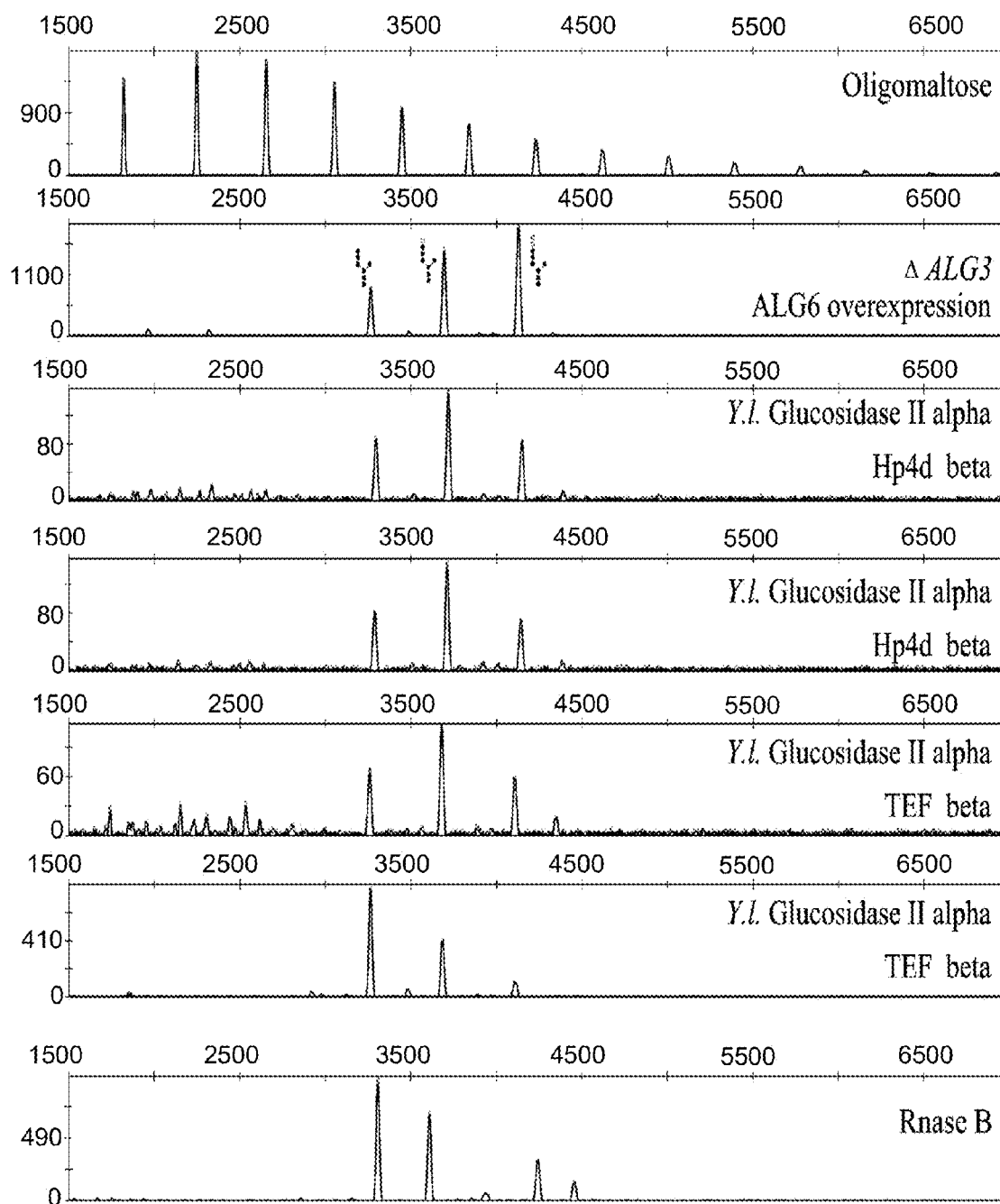


FIG. 33

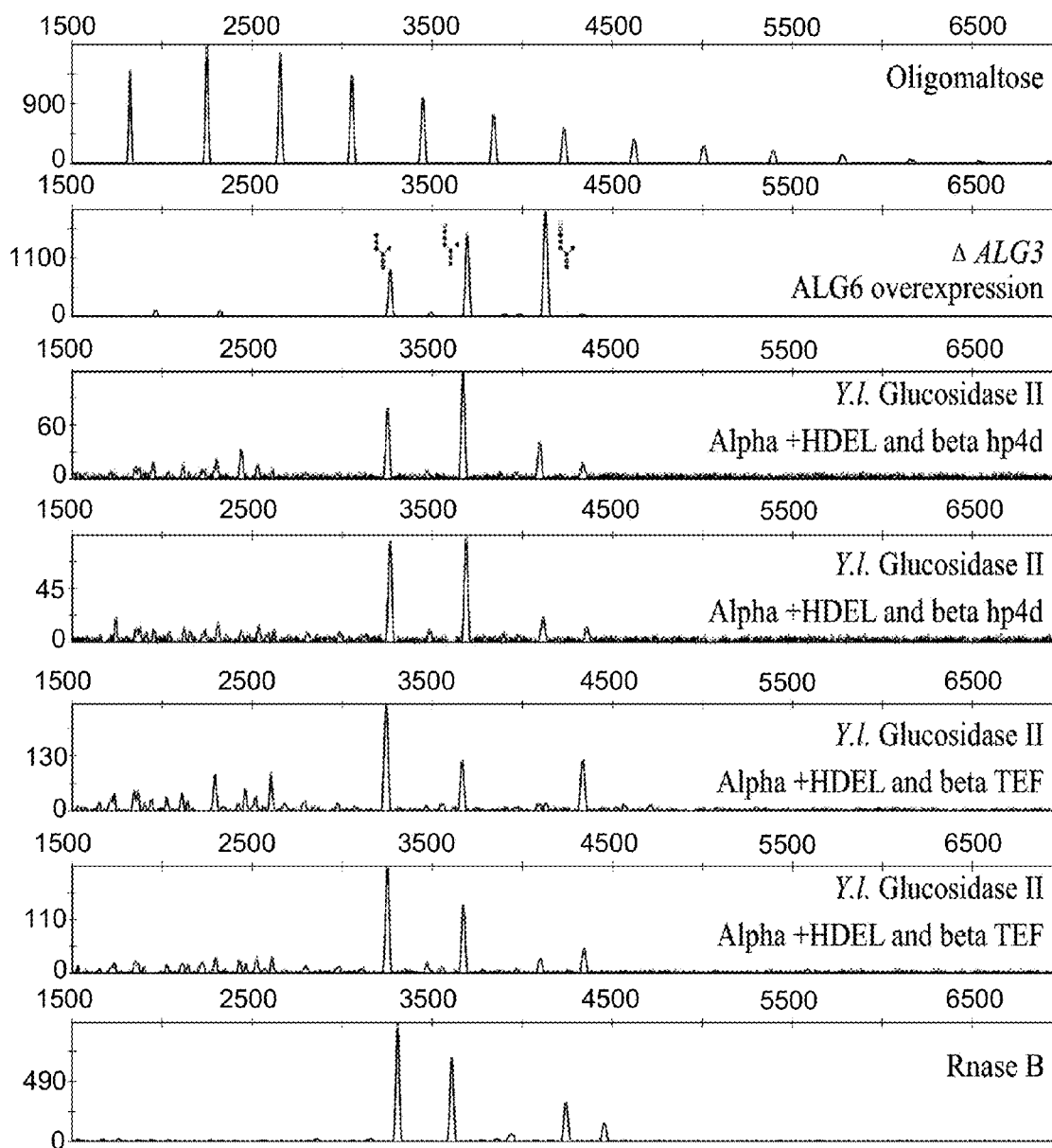


FIG. 34

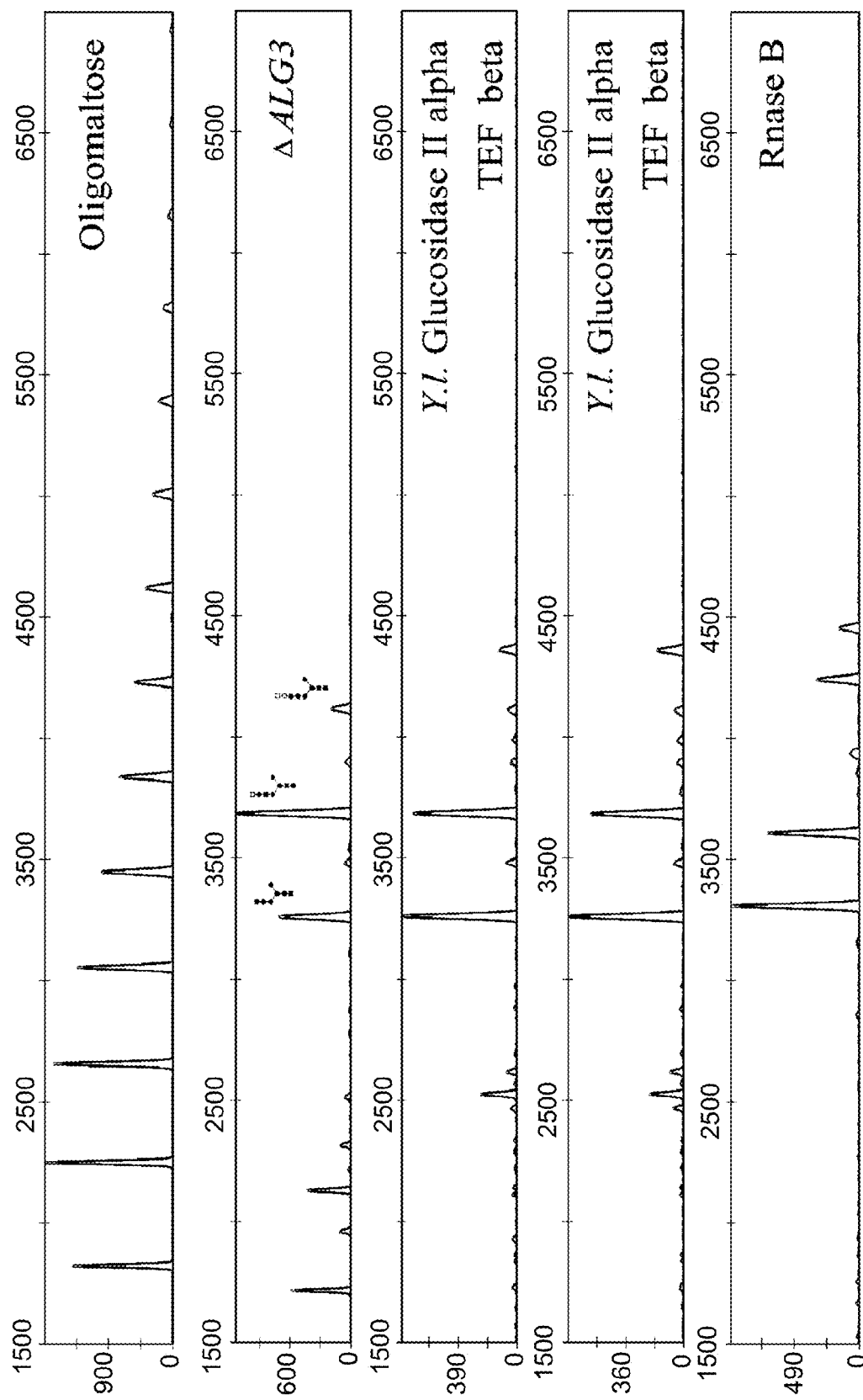


FIG. 35

gtaaagcacgagaactcaagacctgttctcagctctggcttctgtaagcgaaccgagccttcgccgacgacgccgctg  
cccagggctcttcttgggcctctccctacgagctggactcttcttatccagttcaaggacggccagctgcacggcacc  
atctgaagctctgtgtctcccaacgagaaggtgaagctgcccttggtggtgtcttctctggagtctggcgccgctcgagt  
gggtggtggacgaggagaagcgaatgaacggcgacatccagctgcgacacgactctaaggcccgaaggagcgata  
caacgaggccgagaagtggtgctggtcggcgccctggagctgtctaagaccgccacctgcgacccgagaccga  
gtctggcttcacccgagtgtgtacggccccgacaaccagttcgaggccgtgatccgacacgcccccttcttgcggac  
ttcaagcgagatggccagaccacgtgcagctgaacaacaagggctacctgaacatggagcactggcgacccaagg  
tggaggtggagggcgaggggtgagcagcagaccaggaggacgagctacctgggtgggacgagcttctcgccggca  
acaccgacaccaagccccgagggcccgagctgtgtgggcttgacatcaccttccccggctacaagcagtggttcggc  
atccccgagcacgccgactccctgtctctgaaggagaccgaggcgccgaggggcaaccacgaggagccctaccga  
atgtacaacgccgacgtgttcgagtacgagctgtccttctccatgacctgtacggcgccatccccctcatgcaggccca  
ccgaaaggactctaccgtgggctgttctggctgaacgccgccgagacctgggtggacatcgtgaagtctaccttctt  
cccaaccccttgccctgggctgggagccaccacgacaccagttcacttggttctctgagltctggccagctggac  
gtgttcgtgttctgggccccacccccaggagatttctaagacctacggcgagctgacggctacacccagctgcccc  
agcacttcgccattgcctaccaccagtgctgatggaactacatcaccgacgaggacgtgaaggaggtggaccgaaact  
tcgacaagtaccagatccctacgacgtgatctggctggacatcgagtacaccgacgaccgaaagtacttcacctggg  
acccctgtcttccccgacccatctctatggaggagcagctggacgagctctgagcgaaagctggctgtgatcatcga  
ccccacatcaagaaccaggacaagtactctatcgtcgaggagatgaagtetaaggacctggccaccaagaacaagg  
acggcgagatttacgacggctggtgttggccggctcttctcactggatcgacacctcaaccccgctgccatcaagt  
gtgggtgtctctgttcaagttcgacaagtcaagggcacctgtctaactgttcatctggaacgacatgaacgagccctc  
tgtgtcaacggccccgagaccaccatgccaaggacaacctgcaccacggcaactgggagcaccgagacatccac  
aacgtgcacggcatcacctggtgaacgccacctacgacgccctgctggagcgaagaagggcgagatccgacgac  
cctcatctgacccgatcttactacgccggtgcccagcgaatgtctgccatgtggaccggcgacaaccaggccacctg  
ggagcacctggccgctctatccccatggtgctgaacaacggaatcgccggcttccccctcgccggtgccgacgtggg  
cggttcttccagaacccctctaaggagctgtgacccgatggtatcaggccggcatctggtatcttcttccgagccca  
cgccacatcgacaccgacgacgagagccctacctgatcgccgagccccaccgatctatcatctcagggcatccg  
actgcgataccagctgtgtcccgcctgttacaccgccttccacgaggcctctgtgaacggcatgcccacgtgcgacc  
ccagtactacgcccacccctgggacgaggccggcttcgcatcgacgaccagctgtacctgggctctaccggcctgt  
ggccaagcccgtggtgtctgaggaggccaccaccgcccacatctacctggctgacgacgagaagtactacga

FIG. 36A

ctacttcgactacaccgtgtaccagggcgctggcaagegacacaccgtgcccgcctccatggagaccgtgccctg  
ctgatgcagggcgccacgtgatccccgaaaggaccgaccccgacgattcttgcctgatgcgatgggacccct  
acaccctggtggtggtgctggacaagaacggccaggccgacggctctctgtacgtggacgacggcgagaccttga  
ctacgagcgaggcgctacatccatcgacgattccgattccaggagtctgccctggtctccgaggacgtgggcacca  
agggccccaagaccgccgagtacctcaagaccatggccaacgtgagtgaggcgagtggtggtcgtggaccccc  
ccaaggagtggcagggcaagacctctgtgaccgtgatcgaggacggcgccctctgccgcctctaccgcctccatgca  
gtaccactctcagcccgacggcaaggccgcctacgccgtggtgaagaaccccaacgtgggcacggcaagacctg  
gcgaatcgagttctaatagcctag

FIG. 36B

agcgcctggcgacgcctcttctcgacccccgagcgctgggccccgagttcgccaagttctacaaggacaccaccacc  
tteacctgtatctctcaccgcccatccagattcccttctctgccgtgaacgacgactactgtgactgtcccgacggct  
ctgacgagccccggcacctctgcctgtgcctttctgtctcgaaactctgccctgacccccggtagcgacccggatct  
gacgacctggagctgacctctgccctgcccggttctactgtgaagaacaagggccacaagccccggctacgtgccc  
ttccagcgagttaacgatggcatctgtgactacgagctgtgttgtagcgatcgatgagtggtcgacccggcg  
gaaccaagtgtgaggacaagtgtgaaggagatcggaaggagtggtcgaaagaaggaggagaagcgacagaagt  
ctatgaccgccgctctgaagaagaagaaggacctgctggtcgaggccggacgacagcagaaggaggtcgagga  
caacatcaagcgactggaggtggagatccaggcccaggagctgaaggtcaacgacctgcaggccgagctggag  
gaggtggagcagcaggaggcctctaaggtcgtaagggcaagaccgccggcaaggtgaacgtgctggctggcc  
tcgccaagtctcgagtggaggagctgcgaaacgccctgatggacgtgcgaaaggagcgagatgacaccggagc  
ccgagttaaggaaactgaagagatcctgtctaagttcaaggtggagtacaacccaactcaacgacgagggcgtg  
aagcgagccgtgcgatcttgggaggactacgccgccaagggcacacctggagggcgccgtgaacaacgccag  
gaccgagacctggacgagatcgccaagccccgacgacgagaaggccggcatcaactgggagcagtgaggagaac  
gaggaggacggctgtgaggtggcctggtgtaccagctggccgctacctgccccctctctggtggagttcatcg  
agggcaaggtgctgttcgtcgaggcctgctggaggacaacggcatcctgccaaggccgagacctctacc  
tctgagtctaaggtggtgtctgaggccccgagaggccgtgaaggtctccgagaaggagctgggcgacaagcagaa  
gcagctgaaggaccacaagtctgacctggagaccgactacggcggtgggtctatcttccgagccctgaagggcgt  
gtgtatctctaaggactctggcgagtacacctacgagcactgtttctggaccagaccaagcagatccccaaagg  
gcggaggctctacccgaatgggcaagtacaccggcatcggtctgtgtctgtggacgtgctgaacgaggccggc  
gagatcgctgcctgaggatcgagtgacctgcagtacgccaacggccagggtgttgaacggacccgcccgatc  
taccaccgtgatcctgacctgtggcgaggaggacgccatcctgaaggtggccgaggacgagaagtgtgtgtactc  
tatgcacgtgacctctcccggcgtgtgtcccgcggtgacgaggcgccaccgcccccaaccgaaaggacgagc  
tgtaatagccta

FIG. 37

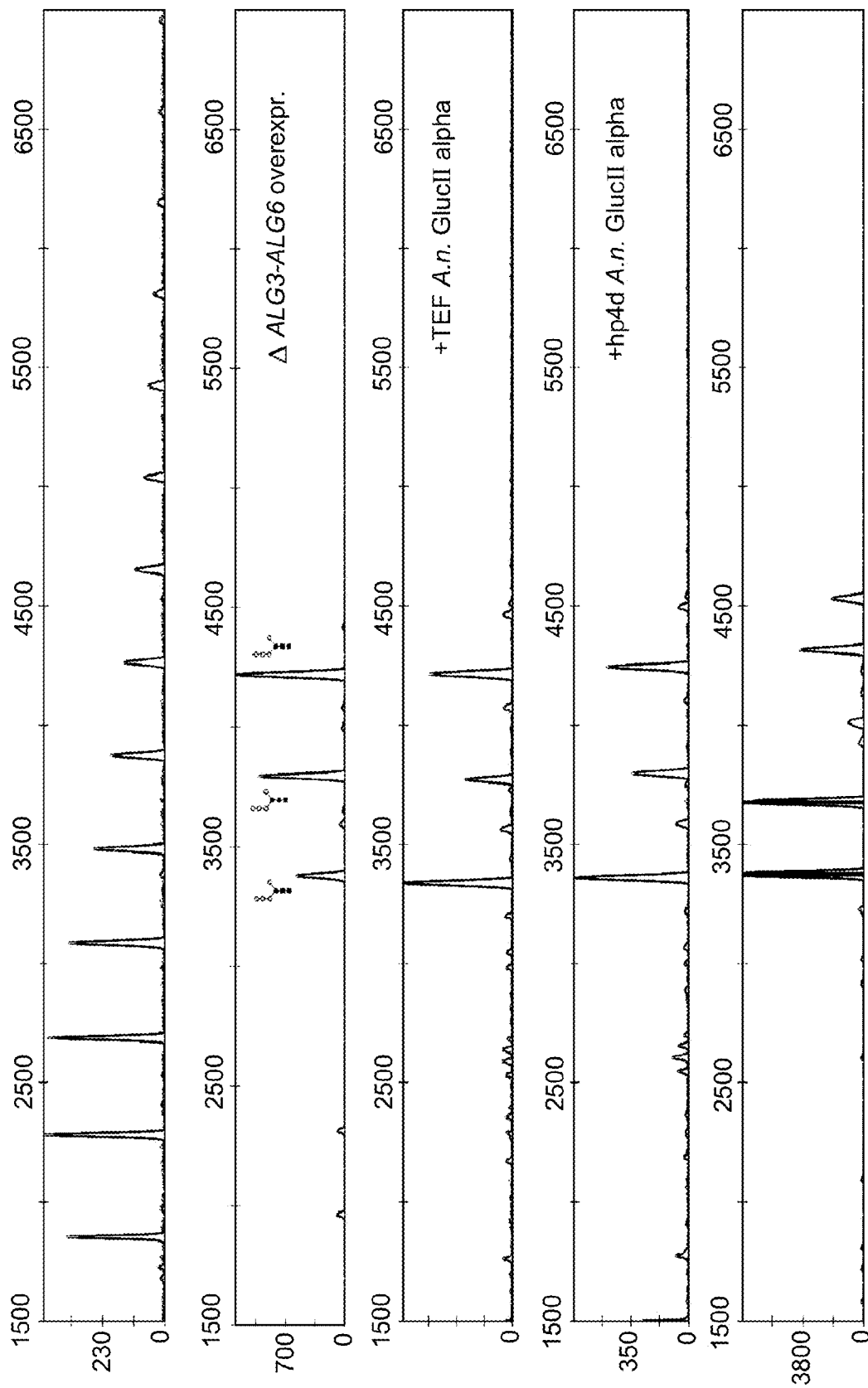


FIG. 38

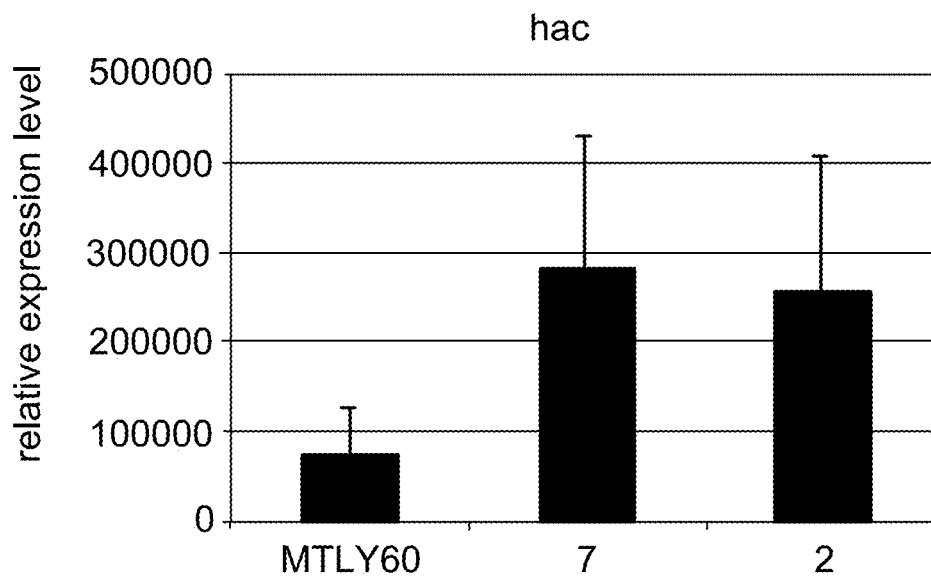


FIG. 39A

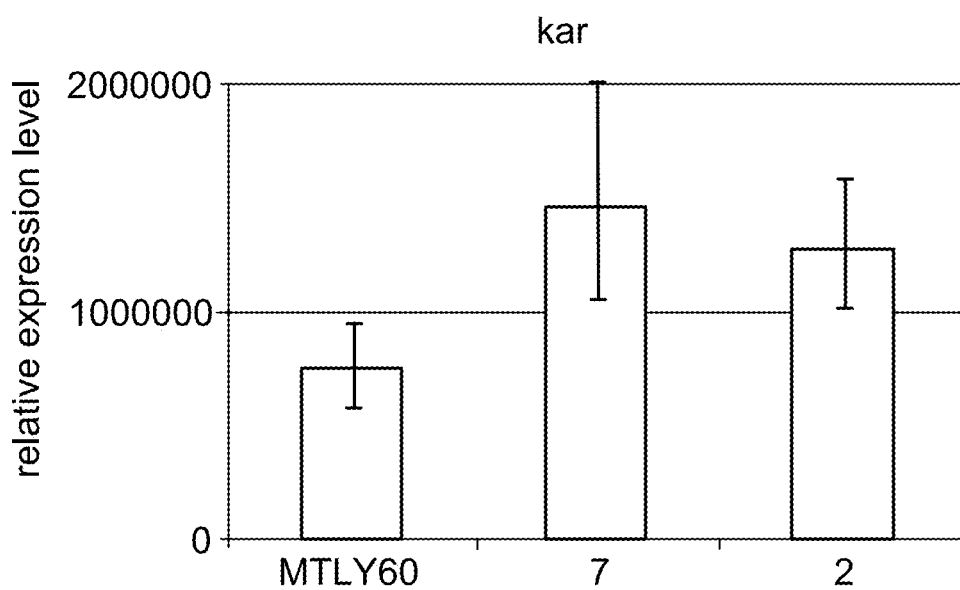
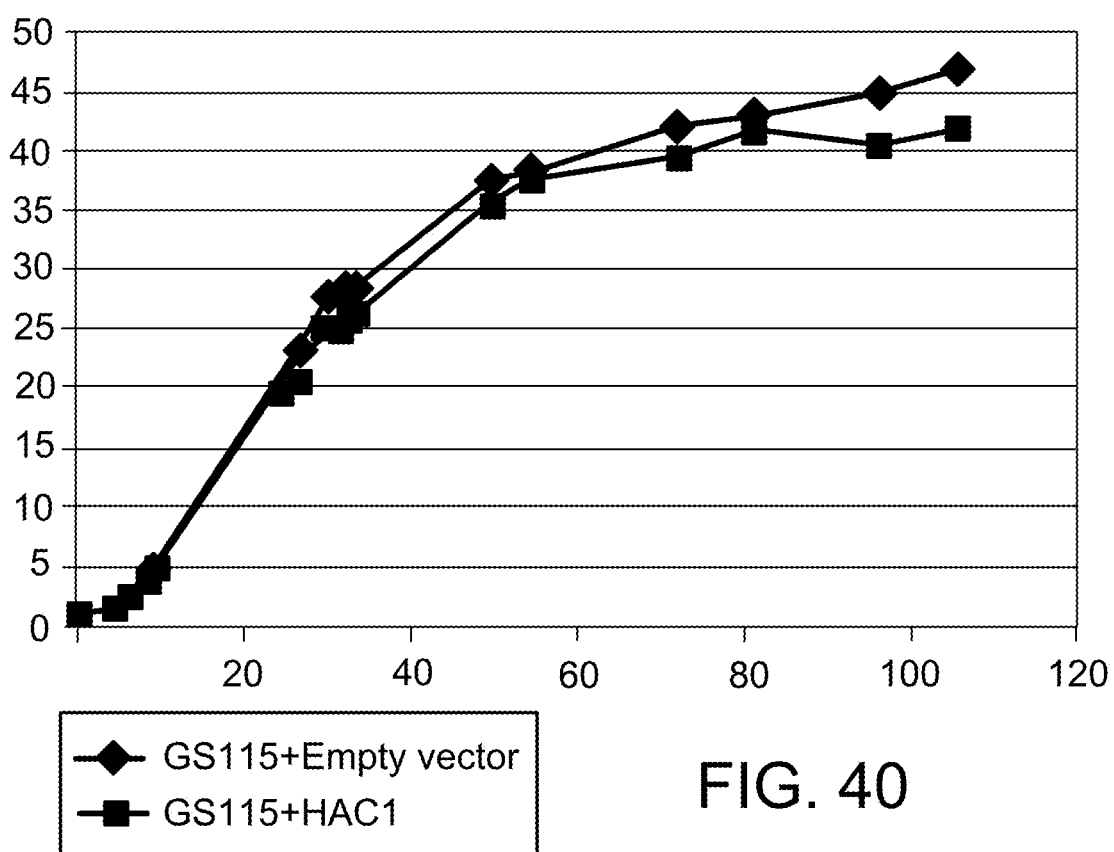


FIG. 39B



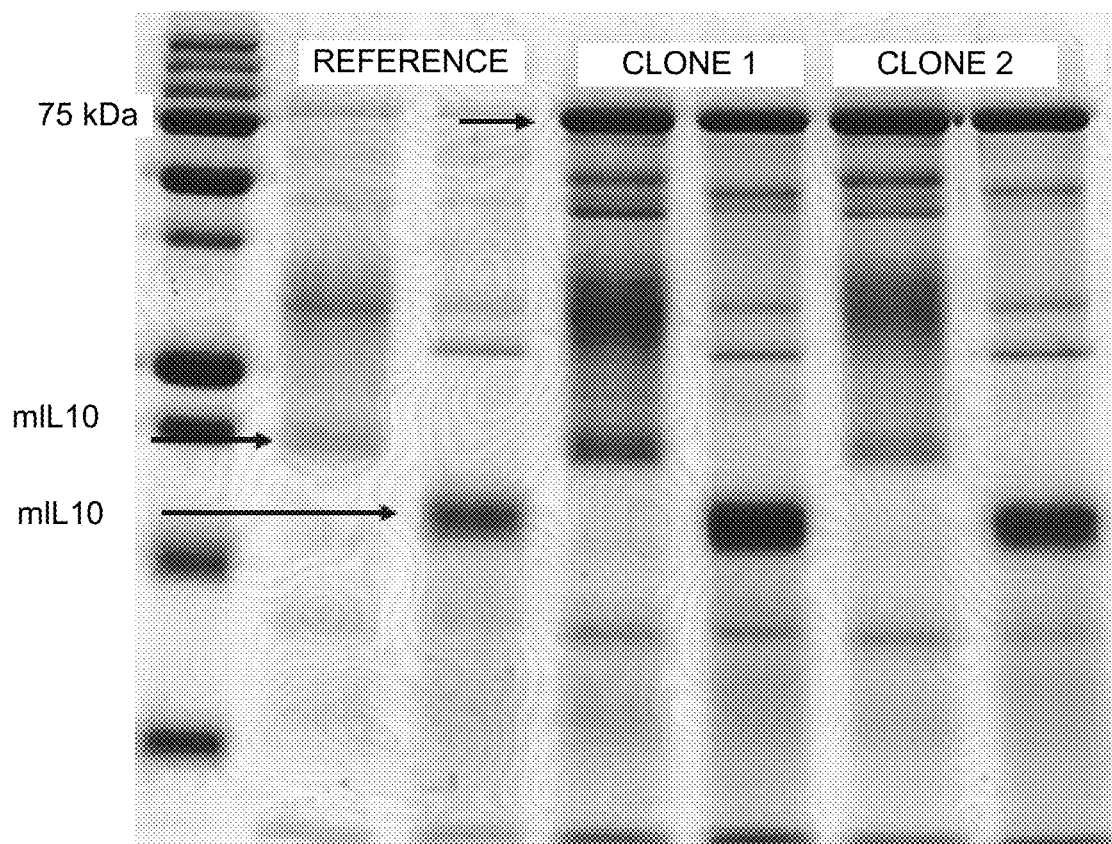


FIG. 41

gagctcagcgctaccaagcgaggctctcccaacccacccgagccgcccgtgtgaaggccgcttcagacc  
tcttggaacgcctaccaccacttcgecttccccacgacgacctgcaccccgtgtctaactcgttcgacgacgag  
cgaaacggctggggctcttctgccatcgacggcctggacaccgccatcctgatggcgacgccgacatcgtga  
acaccatcctgcagtacgtgccccagatcaacttcaccaccaccgccgtggccaaccagggeatctctgtgttcg  
agaccaacatccgatacctgggcggcctgctgtctgcctacgacctgctgcgaggcccccttcttctctggcca  
ccaaccagaccctgggtgaactctctgctgcgacaggcccagaccctggccaacggcctgaagggtggctttacc  
accccccttggegtgcccgaacccacccgtgttttcaacccacccgtgcgacgatctggcgcttcttaacaac  
gtggccgagatcggtctctgtgtgctggagtggacccgactgtctgacctgaccggcaacccccagtacgcc  
agctggcccagaaggcgagctcttacctgtgaaccccaagggtctctccgaggcctggcccgactgatcg  
gcaccttcgtgtctaccttaacggcaccttcaggactcttccggctcttggcttgccctgatggactctttctacg  
agtacctgatcaagatgtacctgtacgaccccggtgccttcgcccactacaaggaccgatgggtgctggccgcc  
gactctaccatgcccacctggcctctcaccctctacccgaaaggacctgaccttctgtctcttacaacggcc  
agtctaccttcccactctggacacctggcttcttcgcccgtggcaacttcactcctggcgccatcctgctgaa  
cgagcagaagtacatcgacttcggcatcaagctggccttcttacttcgccacctacaaccagaccgcctctgg  
catcggccccgagggttcgcctgggtggactctgtgaccggcgctggcggtctcccccttctctcagtctgg  
cttctactcttctgcggcttctgggtgaccgccccctactacatcctgcgacccgagaccctggagtctctgtact  
acgcctaccgagtaccggcgactctaaagtggcaggacctggcctgggaggccttctctgccatcgaggacgc  
ctgtcgagccggtctgcctactcttctatcaacgacgtgaccaggccaacgggtggcgagcctctgacgaca  
tggagtcttctggttcgcccaggccctgaagtacgcctacctgatcttcgccgaggaatctgacGtgcagggtgc  
aggccaacggcggaacaagtctgtgtcaacaccgaggccccaccccttctctatccgatcttctctcgacgag  
gcggccacctggcccatgacgagctgtaatagcctaggtacc

FIG. 42

cggcggactgcgtccgaaccagctccagcagcgTTTTccgggccattgagccgactgcgaccccgccaacgtgtct  
tgccccacgcactcatgtcatgttggtgttgaggccactTTTtaagtagcacaaggcacctagctcgagcaaggtgt  
ccgaaccaaagaagcggctgcagtgggtgcaaacggggcggaacggcgggaaaaagccacgggggcacgaatt  
gaggcacgccctcgaatttgagacgagtcacggccccattegcccgcgcaatggctcgccaacgcccggtcttttgca  
ccacatcagggtaccccaagccaaaccttTgtttaaaaagcttaacataattataccgaacgtagggttgggcgggcttgc  
tcgctctgtccaaggcaacatttatataagggctctgcatcgccggctcaattgaatctTTTTcttctctctctatattcatt  
cttgaattaaacacacatcaaca

FIG. 43

cttatctggggcagtgaaagtatatgttatggtaatagttacgagttagttgaacttatagatagactggactatacggetatc  
ggtccaaattagaaagaacgtcaatggctctctgggcggaattcgtataacttcgtatagcaggagttatccgaagcgat  
aattaccctgttatccctagcttatcgatacgcgtgcatgctgaggtgtctcacaagtgccgtgcagtcgccgccccactt  
gcttctctttgtgtgtagtgatctacgtacattatcgagaccgttggtcccgcccacctcgatccggcatgctgaggtgtctcaca  
agtgccgtgcagtcgccgccccacttgcttctctttgtgtgtagtgatctacgtacattatcgagaccgttggtcccgcccacctc  
gatccggcatgctgaggtgtctcacaagtgccgtgcagtcgccgccccacttgcttctctttgtgtgtagtgatctacgtacatt  
atcgagaccgttggtcccgcccacctcgatccggcatgctgaggtgtctcacaagtgccgtgcagtcgccgccccactt  
gcttctctttgtgtgtagtgatctacgtacattatcgagaccgttggtcccgcccacctcgatccggcatgcatgacgggc  
aaaagtgcgtatatatacaagagcgttgcagccacagatttccactccacacaccacatcacatacaaccacacac  
atccacgtgggaacccgaaactaaggatccatgaagcttccaccatctcttcacagcctgcgctaccctggcgctac  
caagcgaggtctcccaaccccacccgagccgccgtgtgaaggccgcttccagacctttggaacgcctaccacc  
acttgccttccccacgacgacctgcaacccgtgtctaaactcgttcgacgacgagcgaaacggctggggtcttctgc  
catcgacggcctggacaccgccatcctgatggcgacgccgacatcgtaacacacatcctgcagtacgtgccccaga  
tcaacttcaccaccaccgccgtggccaaccagggcatctctgttctgagaccaacatccgatacctggggcgccctgct  
gtctgcctacgacctgctgcgagggcccccttctcttctctggccaccaaccagaccctgggtgaactctctgctgcgacagg  
cccagaccttgccaacggcctgaaggtggcttttaccacccccctctggcgtgcccgaacccacgtgttcttcaacc  
caccgtgcgacgatctggcgccctcttctaacaacgtggccgagatcggtctcttggtgctggagtggacccgactgtct  
gacctgacgggcaacccccagtagcccgactggcccagaagggcgagttacctgctgaacccaagggctctcc  
cgaggcctggccccgactgacggcaccttctgtctaccttaacggcaccttccaggactcttccggctcttggctctg  
gcctgatggactctttctacgagtacctgatcaagatgtacctgtacgacccgtggccttcgcccactacaaggaccga  
tgggtgctggccgccgactctaccatgccacctggcctctcaccctctacccgaaaggacctgaccttctgtctctc  
ttacaacggccagctctaccttcccaactctggacacctggcttctctcgcgggtggcaacttcaccttggggcgccatcct  
gctgaacgagcagaagtacatcgacttcggcatcaagctggcctcttctacttcgccacctacaaccagaccgctct  
ggcatcgccccgagggcttcgctgggtggactctgtgaccggcgctggcggtctccccctcttctcagtctggct  
tctactcttctgcccgttctgggtgaccgccccctactacatcttgcgacccgagacctggagctctgtactacgct  
accgagtgaacggcgactctaaagtggcaggacctggcctgggagaccttctctgcatcgaggacgctgtcgagcc  
ggctctgctactcttctatcaacgacgtgacccaggccaacgggtggcgagcctctgacgacatggagctttctggtt  
cgccgagggcctgaagtacgcctacctgatcttcccgaggaatctgacgtgcaggtgcaggccaacggcggaaca  
agttcgtgttaacaccgagggccacccttctctatccgatcttctctcagcagggcgccacctggccccatgacgag  
ctgtaatagcctagggtgtctgtggtatctaagetatcttactcttacaacttctacctcaactatctactttaataatgaa  
tactggtt

FIG. 44A

attctctatgattactgtatatgcgttcctctaagacaaatcgaattccatgtgtaacactcgctctggagagtagtcatccga  
cagggttaactctaattctcccaacaccttattaactctgcgtaactgtaactcttcttgccacgtcgatcttactcaatttctget  
catcatctgctggattgtgtctatcgtctggctetaatacatttattgtttattgcccacaactttcattgcacgtaagtgaatt  
gtttataacagcgttcgccaattgctgcgccatcgctgcggctgtcctaccgttagggtagtgtgtctcacactaccgag  
gttactagagttgggaaagcgatactgctcggacacaccacctgggtcttacgactgcagagagaatcggcggttacctct  
caciaagccctcagtgccggccgggggtgggcgaagaactccagcatgagatccccgcgtggaggatcatccag  
ccggcgctcccgaaacgattccgaagcccaaccttcatagaaggcggtgggaatcgaatctcgtgatggcaggt  
tgggcgtcgttggctggctcatttgaacccagagtcctcgctcagaagaactcgtcaagaaggcgatagaaggcgatg  
cgctgcgaatcgggagcggcgataccgtaaagcagaggaaggcggtcagccattcggcccaagctcttcagcaata  
tcacgggtagccaacgctatgtctgatagcgggtccgccacaccagccggccacagtcgatgaatccagaaaaggc  
ccatttccaccatgatattcggcaagcaggcatcgccatgggtcacgacgagatctcggcgctgggcgatgcgcgctt  
gagcctggcgaacagttcggctggcgagccccgtatgctcttcgtccagatcactgacgacaagaccgggttcca  
tcgagtagctgtcgtcgtatgcgatgttctgcttgggtggcgaatgggcaggtagccggatcaagcgtatgcagccgc  
cgcatcgcacagccatgatggatacttctcggcaggagcaagggtgagatgacaggagatcctgccccggcacttcgcc  
caatagcagccagtccttcccgcttcagtgacaacgtcgagcacagctgcgcaaggaaacgcccgtcgtggccagcca  
cgatagccgcgtgcctcgtctgcagttcattcagggcaccggacaggtcggtcttgacaaaaagaaccggggcgcccc  
tgcgtgacagccggaacacggcggcatcagagcagccgattgtctgttgtgcccagtcatagccgaatagccttcca  
cccaagcggcgaggagaacctgcgtgcaatccatttgttcaatcatgcgaaacgatcctcatcctgtctcttgatcagatctt  
gateccctgcgccatcagatccttggcggcaagaaagccatccagtttactttgcagggttcccaaccttaccagagggc  
gccccagctggcaattccgggtcgttgcgttccataaaaccgcccagctatagctatcgccatgtaagcccactgcaagct  
acctgcttctcttgcgttgcgttcttcccttgcagatagccagtagctgacattcaccggggtcagcaccgttctgcg  
gactggcttctacgtgttccgttcccttagcagcccttgcgcccagtgcttgcggcagcgtgaagctagcttatgcggt  
gtgaaataccgcacagatgcgtaaggagaaaaataccgcatcaggcgctcttccgcttctcgtcactgaactcgtcgtcgt  
cggctcgttcggctgcggcgagcgggtatcagctcactcaaaaggcggtataacggttatccacagaatcaggggataacgc  
aggaaagaacatgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaaggccggttgcgtggcggttttccatag  
gtcgcgccccctgacgagcatcacaaaaatcgacgctcaagtcagagggtggcgaaacccgacaggactataaagata  
ccaggcggttccccctggaagctccctcgtgcgctctcctgttccgacctgcccgttaccggataacctgtcgccttctcc  
cttcgggaagcgtggcgcttctcatagctcacgctgtaggtatctcagttcgggtgtaggtcgttcgtccaagctgggctgt  
gtgcacgaacccccgttcagcccgaccgtgcgccttatccggttaactatcgtcttgagccaacccggtaagacacga  
cttatcgccactggcagcagccactggtaacaggattagcagagcgaggatgtagggcggtgtacagagttcttgaagt  
gggtggcctaactacggctacactagaaggacagtatttggatatctgcgctctgctgaagc

FIG. 44B

cagttaccttcggaanaagagttggtagctcttgatccggcaaaaccaccgctggtagcggcggtttt  
tgtttgaagcagcagattacgcgcaaaaaaaggatcacaagaagatccttgatctttcttactgaacg  
gtgatccccaccggaattgcggccgctgtcgggaaccgcgttcaggtggaacaggacaccteccttgca  
ttcttggtatatcagtataggctgatgtattcatagtgggggttttcataataaattactaacggcaggcaacatt  
cactcggcttaaacgcaaaacggaccgtcttgatatctctgacgcattgaccaccgagaaatagtgttagtt  
accgggtgagttattgttctctacacaggcgacgcccatcgtctagagttgatgtactaactcagatttca  
acctacctatccctggtacgcacaaagcactttgctagatagagtcgacaaaggcgggccccccctcga  
gattacctgttatccctacataacttcgtatagcatacattatacgaagttattctgaattccgagaaacaca  
caacatgccccattggacagacatgcggatacacaggttgtgcagtaccatacactcgcagacagcagg  
tcgtctgaccatcatacaagctgaacagcgcctccatacttgacgcctctctatatacacagttaaattacatat  
ccatagtctaacctctaacagttaattcttctggttaagcctccagccagccttctggtatcgttggcctctc  
aataggatctcgggtctggccgtacagacctcggccgacaattatgatatccgttccggtagacatgacatc  
ctcaacagttcggtagtctgtcgcgagagcgtctcccttgctcgaagaccaccccgggggtcagaataa  
gccagtcctcagagtcgcccttaggtcgggtctgggcaatgaagccaaccacaaactcgggggtcggatcg  
ggcaagctcaatggtctgcttgagtagtctgccagtgccagagagcccttgcaagacagctcggccag  
catgagcagacctctggccagcttctcgttgggagaggggactaggaactccttgtagtgggagttctcgta  
gtcagagacgtctcctctctgttcagagacagtttctcggcaccagctcgcaggccagcaatgattcc  
ggttccgggtacaccgtgggcgttggatgacggaccactcggcgattcgggtgacaccggtagtgggtct  
tgacagtgttgccaatatctgcgaactttctgtctcgaacaggaagaaaccgtgcttaagagcaagttcctt  
gagggggagcagagtgccggcgtaggtgaagtcgtcaatgatgctgatgggtcttgatcatgcacacat  
aaggtccgaccttatcggcaagctcaatgagctccttggtggtgtaacatccagagaagcacacaggttg  
gtttcttggtgctgccacgagcttgagcactcgagcggcaaaaggcggacttgtaggacgttagctcgagctc  
gtaggagggcattttggtggtgaagaggagactgaaataaatttagtctgcagaacttttatcggaac

FIG. 44C

tagcaggagttatccgaagcgataattaccctgttatccctagcttatcgatacggcggactgcgtccgaaccagctcca  
gcagegtttttccgggccattgagccgactgcgaccccgccaacgtgtcttggcccacgcactcatgtcatgttggtgtt  
gggaggccactttttaagtagcacaaggcacctagctcgcagcaagggtgtccgaaccaaagaagcggctgcagtgg  
gcaaacggggcggaacggcgggaaaaagccacggggggcacgaattgaggcacgccctcgaatttgagacgagt  
caeggccccattcgcccgcgcaatggctcgccaacgcccggcttttgcaccacatcaggftaccccaagccaaacctt  
tgtgtaaaaagcftaacatattataccgaacgtagggttgggggggcttgcctcgtctgtccaaggcaacattatataag  
ggctcgcacgcgggtcaattgaatctttttcttctctctctatattcattcttgaattaaacacacatcaacaggatcc  
atgaagctttccaccatctcttcacagcctgcgctaccctggcgcctaccaagcgaggctctcccaacccccaccgag  
ccgccgctgtgaaggccgcttcagacctcttgaacgcctaccaccacttcgcttccccacgacgacctgcacc  
ccgtgtctaactcgttcgacgacgagcgaaacggctgggctcttctgccatcgacggcctggacaccgccatcctgat  
gggcgacgccgacatcgtgaacaccatcctgcagtacgtgccccagatcaacttcaccaccaccgccgtggccaacc  
agggcatctctgtgttcgagaccaacatccgatacctggcgggcctgctgtctgcctacgacctgctcgaggccccctt  
cttctcttgccaccaaccagacctggtgaactctctgctgcgacagggccagacctggccaacggcctgaagg  
ggctttaccacccccctetggcgtgcccgaccccaccgtgttcttcaaccccaccgtgcgacgatctggcgctcttcta  
acaacgtggccgagatcggtctctgtgtgctggagtggaccgactgtctgacctgaccggcaacccccagtaagcc  
cagctggcccagaagggcgagtcttacctgctgaaccccaagggtctcccaggcctggccccgactgatcgcca  
ccttcgtgtctaccttaacggcaccttcaggactcttcggctcttggcttgccctgatggactctttctacgagtacctg  
atcaagatgtacctgtacgacccccgtggccttcgcccactacaaggaccgatgggtgctggccgcccactctaccatc  
gcccacctggcctctacccccctacccgaaaggacctgaccttctgtctcttacaacggccagctctaccttcccaa  
ctctggacacctggcttccttcgcccgtggcaactcatctggcgggcatcctgctgaacgagcagaagtacatcgac  
ttcggcatcaagctggcctcttctacttcgccacctacaaccagaccgctctggcatcgcccccaggggcttcgct  
gggtggactctgtgaccggcgctggcggtctccccctcttctcagcttggttctactcttctgccggcttctgggtga  
ccgccccctactacatctgcgacccgagacctggagtctctgtactacgcctaccgagtaccggcgactctaagt  
gcaggacctggcctgggaggccttctctgccatcgaggacgccctgctgagccggctctgctactcttctatcaacgac  
gtgaccaggccaacgggtggcgagcctctgacgacatggagtcttctggttcgcccaggccctgaagtacgcctac  
ctgatcttcgccgaggaatctgacgtgcaggtgcaggccaacggcggaacaagtctgttcaacaccagggccca  
ccccctctctatccgatcttctctcagcagggcgccacctggcccatgacgagctgtaatagcctagggtgtctgtgt  
atctaagctatttatcactctttacaactctacctaactatctttaaataatgaatatcgtttattctctatgattactgtata  
tgcgttctctaaagacaaaatcgaattccatgtgtaacactcgctctggagagttagtcacccgacagggttaactctaatec  
ccaacaccttattaactctcgtaactgtaactcttcttgcacgtcgatcttactcaatttctgctcatcatctgctggattg  
ttgtctatcgtctggt

FIG. 45A

ctaatacatttattgtttattgcccacaactttcattgcacgtaagtgaattgtttataacagcgttcgccaattgctgcgc  
catcgtcgtccggctgtcctaccgttagggtagtgtgtctcacactaccgaggttactagagttgggaaagcgatactgc  
ctcggacacaccacctggtcttacgactgcagagagaatcggcggttacctcctcacaagccctcagtgcggccgcc  
cgggggtgggcgaagaactccagcatgagatccccgcgtggaggatcatccagccggcgtcccggaacgatlc  
cgaagcccaacctttcatagaaggcggcggtggaatcgaaatctcgtgatggcaggttgggcgtcgttggtcgtgca  
tttcgaaccccagagtcccgctcagaagaactcgtcaagaaggcgatagaaggcgatgcgtgcgaatcgggagcg  
gcgataccgtaaagcacgaggaagcggtcagcccattcgccccaagctcttcagcaatatcacgggtagccaacg  
ctatgtcctgatagcggtcgccacaccagccggccacagtgcgatgaatccagaaaagcgccattttccaccatga  
tattcggcaagcaggcatcgccatgggtcacgacgagatcctcgccgtgggcatgcgcgcttgagcctggcgaac  
agttcggctggcgagccccctgatgtcttcgtccagatcactcgtatgcacaagaccggttccatccgagtacgtg  
ctcgtcgtatgcgatgtttcgttgggtggaatgggcaggttagccggatcaagcgtatgcagccgcgcattgcac  
agccatgatggatactttctcggcaggagcaaggtgagatgacaggagatcctgccccggcacttcgcccatagca  
gccagtccttccccgttcagtgcacaacgtcgagcacagctgcgcgaaggaaacgcccgtcgtggccagccacgatag  
ccgcgtcgtcctcgtcctgcagttcattcagggcaccggacaggtcggcttgcacaaaagaaccgggcccctcgcg  
ctgacagccggaacacggcggcatcagagcagccgatgtgtgtgtgccagtcatagcgaatagcctctccacc  
aagcggccggagaacctgcgtgcaatccatcttgttcaatcatgcgaaacgacccatcctgtctcttgatcagatcttg  
atccccctgcgcatcagatccttggcggcaagaaagccatccagtttacttgcagggttcccaaccttaccagaggg  
cgccccagctggcaattccggttcgcttgcgtgtccataaaaccgcccagctctagctatcgccatgtaageccactgca  
gtacctgtcttctcttgcgttgcgttttcccttgcagatagcccagtagctgacattcatccggggtcagcaccgttt  
ctcgggactggctttctacgtgttcgcttccctttagcagcccttgcgcccgtagtgcttgcggcagcgtgaagctagctt  
atcgggtgtgaaataccgcacagatgcgtaaggagaaaaataccgcatcaggcgtcttccgcttctcgtcactgaet  
cgctgcgctcggctgttcggctgcggcgagcggatcagctcactcaaaggcggtaatacgggttatccacagaatcag  
gggataacgcaggaaagaacatgtgagcaaaaggccagcaaaaggccaggaaaccgtaaaaaggccgcgttgctg  
gcgtttttcataggtccgccccctgacgagcatcacaaaaatcagcgtcaagtcagaggtggcgaaacccgac  
aggactataaagataccaggcggttccccctggaagctccctcgtgcgctctcctgttccgacctgcccgttaccggat  
acctgtccgcttttctccctcgggaagcgtggcgctttctcatagctcacgctgtaggtatctcagttcggtgtaggctgt  
tcgctccaagctgggctgtgtgacgaacccccgttcagcccagccgtgcgccttatccggtaactatcgtcttgag  
tccaacccggtaagacacgacttatcgccactggcagcagccactggtaacaggattagcagagcgaggtatgtagg  
cgggtctacagagttctgaagtgggtggcctaactacggctacactagaaggacagtatttggtatctgcgctctgctga  
agccagttaccttcggaaaaagagtggtagctcttgatccggcaacaaaccaccgctggtagcggcggtttttgttt  
gcaagcagcagattacgcgcagaaaa

FIG. 45B

aaaggatctcaagaagatcccttgatctttcttactgaacgggtgatccccaccggaattgcggccgctgctgg  
gaaccgcgttcaggtggaacaggacacctccctgcacttcttggtatatcagtataggctgatgtattcatag  
tgggggttttcataataaaatttactaacggcaggcaacattcactcggcttaaaccgaaaacggaccgtcttga  
tatcttctgacgcattgaccaccgagaaatagtgttagttaccgggtgagttattgttcttctacacaggcgacg  
cccatcgtctagagttgatgtactaactcagatttcactacctaccctatccctgggtacgcacaaagcaactttgc  
tagatagagtcgacaaaggcggggccccccctcgagattaccctgttatccctacataacttcgtatagcatac  
attatacgaagttattctgaattccgcccagagagccattgacgttctttctaatttgaccgatagccgtatagt  
ccagtctatctataagttcaactaactcgtaactattaccataacatatacttcaactgccccagataaggttccga  
taaaaagttctgcagactaaatttatttcagtctctcttcaccacaaaatgccctcctacgaagctcgagcta  
acgtccacaagtcgcctttgccgctcgagtgtcaagctcgtggcagccaagaaaaccaacctgtgtgctt  
ctctggatgttaccaccaccaaggagctcattgagcttgccgataaggtcggaccttatgtgtgcatgatcaa  
gaccatatacgaatcattgacgaacttcacctacgccggcactgtgtccccctcaaggaaacttgctcttaag  
cacggtttcttctgttcgaggacagaaagttcgcagatattggcaacactgtcaagcaccagtaccgggtgc  
accgaatcgcgagtggtccgatatcaccaacgcccacgggtgtaccgggaaccggaatcattgctggcct  
gcgagctggtgccgaggaaactgtctctgaacagaagaaggaggacgtctctgactacgagaactcccag  
tacaaggagttcttagtccccctctcccaacgagaagctggccagaggtctgctcatgctggccgagctgtct  
tgcaagggtctcttgccactggcgagtactccaagcagaccattgagcttgccgatccgaccccgagttt  
gtggttggttcattgcccagaaccgacctaaaggcgactctgaggactggcttattctgacccccgggggtg  
ggtcttgacgacaaggagacgctctcggacagcagtaccgaactgttgaggatgtcatgtctaccggaac  
ggatatcataattgtcggccgaggtctgtacggccagaaccgagatccattgaggaggccaagegataacc  
agaaggctggctgggagggttaccagaagattaactgttagagggttagactatggatatgtaatttaactgtgt  
atatagagagcgtgcaagtatggagcgtgttcagcttgatgatggtcagacgacctgtctgatcgagtatg  
tatggtactgcacaacctgtgtatccgcatggtctgtccaatggggcatgttgtgtgtttctcggaaattcgata  
acttcgta

FIG. 45C

caaggggtaggcgaatggtacgattccgccaagtgagactggcgatcgggagaaggggttggtggtcatgggggata  
gaatttgtaagtggaaccactacgagtagcgatttgataccacaagtagcagagatatacagcaatggtggg  
agtgcagatcgggaatgtactgtacctctgtactcgtactcgtacggcactcgtagaacggggcaatacggggga  
gaagcgatcgccgtctgttcaatgccacaagtcggagtaatgctcgagtatgaagtcctgtacctccctgtcaatca  
tggcaccactgggtctgtactgtattcactggacaagcgccagagttagctagcgaatttcgacctcgacatcac  
cccatcgcagcgacacacatgcccgcacaacagcctctcttattgtagctgaaagtataattgaatgtgaacgtgtacaat  
atcaggtaccagcgggaggttacggccaaggtgataccggaataaccctggcttgagatggtcggtccattgtactg  
aagtgtccgtgtcgtttccgtcactgcccgaattggacatgtttgtttccgatcttcgggcgacctctcctgtctcctgt  
ctgtctcctggactgtgtctaccccatcttttggccctcattggctcctcccgctttcagctcgtctatggttgcatggttt  
cccttatacttttcccacagtcacatgttatggaggggtctagatggacatggtgcaaggcccgagggttgatcgac  
gctttccgcgaaaaaacaagtcacaatacccccgcttattctccctcggtctcgggtatttcacatgaaaactataacct  
agactacacgggcaaccttaacccagagtatacttatafcaaaagggatgggtcctcaaaaatcacacaagcaacg  
gatecatgaagctttccaccatcctcttcacagcctgcgctaccctggccgctaccaagcgaggctctcccaacccca  
cccgagccgcccgtgtgaaggccgcttccagacctcttgaacgcctaccaccacttcgcttccccacgacgac  
ctgcaccccggtcttaactcgttcgacgacgagcgaaacggctggggctcttctgcatcgacggcctggacaccgc  
catcctgatggcgacgcccacatcgtgaacaccatcctgcagtacgtgcccagatcaacttcaccaccaccgccc  
tggccaaccagggcactctgtgttcgagaccaacatccgatacctgggcggcctgtgtctgctacgacctgtctgc  
gaggccccctctctcttggccaccaaccagacctgggtgaactctctgtctgcgacagggccagacctggccaac  
ggcctgaaggtggcttttaccacccccctgtggcgtggcgacccccaccgtgttcttcaacccaccgtgcgacgatct  
ggcgccctcttcaacaacgtggccgagatcggtctctgtgtgctggagtggaaccgactgtctgacctgaccggcaa  
ccccagctacgcccagctggcccagaagggcgagtcttacctgctgaaccccaagggctctcccgaggcctggccc  
ggactgatcggcaccttcgtgtctaccttaacggcaccttcaggactcttccggctcttggcttgacctgatggactct  
ttctacgagtacctgatcaagatgtacctgtacgacccgtggccttcgcccactacaaggacgatgggtgtctggcc  
gccgactctaccatcgcccacctggcctctacccccctacccgaaaggacctgaccttctgtcctcttacaacggcc  
agtctacctctcccaactctggacacctggcttctctcgccgggtgcaacttcacctgggcggcatcctgtctgaacga  
gcagaagtacatcgacttcggcatcaagctggcctcttctacttcgccacctacaaccagaccgctctggcatcgg  
ccccgagggtctgcctgggtggactctgtgacggcgctggcggtctctccccctcttctcagctgtggtcttactctt  
ctgccggcttctgggtgaccgccccctactacatcctgcgacccgagacctggagtctctgtactacgacctaccgag  
tgaccggcgactctaaagtggcaggacctggcctgggaggccttctctgccatcgaggacgacctgtcgagccggtct  
gcctactcttctataacgacgtgacccaggccaacgggtggcgaggacctctgacgacatggagtcttctgttctgcc  
gaggccctgaagtacgacctacctgatcttcgccgag

FIG. 46A

gaatctgacgtgcaggtgcaggccaacggcggaacaagttcgtgttcaacaccgagggccaccccttctctatc  
cgatcttctctcgacgagggcgccacctggcccatgacgagctgtaatagcctaggggtgtctgtggtatctaagct  
atttatcactctttacaacttctacctcaactatctactttaataaatgaatatcgtttattctctatgattactgtatatgcgtt  
cctetaagacaaatcgaattccatgtgtaacactcgtcttgagagttagtcatccgacagggttaactctaattctccc  
aacaccttattaactctgcgttaactgtaactcttcttcccacgtcgatcttactcaattttctgtcatcatctgctggatt  
gttgtctatcgtctggctctaatacatcttattgtttattgcccacaactttcattgcacgtaagtgaattgtttataaca  
gcggttcgccaattgctgcgccatcgtcgtccggctgtcctaccgttagggtagtgtgtctcacactaccgaggttact  
agagttgggaaagcgatactgcctcggacacaccacctgggtcttacgactgcagagagaatcggcggttacctcct  
cacaagccctcagtgccggccgcccggggtggggaagaactccagcatgagatccccgcgctggaggatcat  
ccagccggcggtcccggaaaacgattccgaagcccaacctttcatagaaggcggggtggaatcgaatctcgtg  
atggcaggttggcgctcgttggctggtcatttgaacccagagtcctcgcagaagaactcgtcaagaaggcg  
atagaaggcgatgcgctgcgaatcgggagcggcgataccgtaaagcacgaggaagcggtcagccccattcgcc  
gccaagctcttcagcaatatcacgggtagccaacgctatgtcctgatagcggtcgccacaccagccggccaca  
gtcgtatgaatccagaaaagcggccattttccaccatgatattcggcaagcaggcatcgccatgggtcacgacgag  
atcctcgcgctcgggcatgcgcgcttgagcctggcgaaacagttcggctggcgagccccctgatgctcttcgtc  
cagatcatcctgatcgacaagaccggcttccatccgagtacgtgctcgtcgtatgcgatgtttcgttgggtgga  
atgggcaggttagccggatcaagcgtatgcagccgcgcattgcatcagccatgatggatactttctcggcaggag  
caaggtgagatgacaggagatcctgccccggcacttcgcccataagcagccagtccttcccgttcagtacaa  
cgtcgagcacagctgcgcaaggaacgcccgtcgtggccagccacgatagccgcgctgcctcgtcctgcagtta  
ttcagggcacccggacaggtcggctcttgacaaaaagaaccggcgccccctgcgctgacagccggaacacggcg  
gcatcagagcagccgattgtctgttgcagtcagccgaatagcctctccaccaagcggcgaggagaacct  
gegtgaatccatcttgttcaatcatgcgaaacgatcctcatcctgtctcttgatcagatcttgatcccttgcgccatca  
gatccttggcgggaagaaagccatccagttactttgcagggttcccaaccttaccagagggcgccccagctgg  
caattccggttcgcttgcgttcataaaaaccgcccagctagctatcgccatgtaagcccactgcaagctacctgctt  
tctctttgcgcttgcgtttcccttgcagatagcccagtagctgacattcatccggggtcagcaccgtttctgcgga  
ctggctttctacgtgttccgcttcttttagcagcccttgcgcctgagtgcttgcggcagcgtgaagctagcttatgcg  
gtgtgaaataccgcacagatgcgtaaggagaaaaataccgcacagggcgtcttccgcttctcgtcactgacteg  
ctgcgctcggctgttgcggtgcggcgagcgggtatcagctcactcaaaggcggtataacggttatccacagaatca  
ggggataacgcaggaaagaacatgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaggccgcgttg  
ctggcgttttccataggtccgccccctgacgagcatcacaaaaatcgacgctcaagtcagaggtggcgaaac  
ccgacaggactataaag

FIG. 46B

ataaccaggcggttccccctggaagctccctcgtgcgtctcctgttccgaccctgccgcttaccggataacctgtccgcc  
tttctcccttcgggaagcgtggcgctttctcatagctcacgctgtaggtatctcagttcgggtgtaggtcgttcgctccaag  
ctgggctgtgtgcacgaacccccgttcagcccaccgctgcgccttatccggtaactatcgtcttgagtccaacccg  
gtaagacacgacttatcgccactggcagcagccactggtaacaggattagcagagcgaggtatgtaggcggtgcta  
cagagttctgaagtgggtggcctaactacggctacactagaaggacagtatttggtatctgcgctctgctgaagccagt  
accttcggaaaaagagttggtagctcttgatccggcaaacaccaccgctggtagcggcggtttttgtttgcaagca  
gcagattacgcgcagaaaaaaggatctcaagaagatcctttgatcttttctactgaacgggtgatccccaccggaatt  
ggggccgctgtcgggaaccgcgttcagggtgaacaggacacctcccttcgacttcttggtatatcagtataggctgat  
gtattcatagtgggggttttcataataaattactaacggcaggcaacattcactcggcttaaaccgaaaacggaccgtct  
tgatatctctgacgcattgaccaccgagaaatagtgttagttaccgggtgagttattgttctctacacaggcgacgccc  
atcgtctagagttgatgtactaactcagatttactacctaccctatccctggtacgcacaaagcactttgctagatagag  
tcgacaaaggcgggccccccctcgagattacctgttatccctacataacttcgtatagcatacattatacgaagtatttc  
tgaattccgccagagagccattgacgttctttctaatttggaccgatagccgtatagtccagtctatctataaagtcaact  
aactcgttaactattaccataacatatacttcaactgccccagataagggtccgataaaaagttctgcagactaaatttatttc  
agtcctctctcaccacaaaaatgccctctacgaagctcgagctaacgtccacaagtcgcccttggccgtcagtgcc  
tcaagctcgtggcagccaagaaaaccaacctgtgtgcttctctggatgttaccaccaccaaggagctcattgagcttgc  
cgataaggctcgacattatgtgtgatgatcaagaccataatcgacatcattgacgacttcacctacgccggcactgtg  
ctccccctcaaggaaacttgctcttaagcacgggtttctcctgttcgaggacagaaagttcgcagatattggcaacactgt  
caagcaccagtaccggtgtcaccgaatcgccgagtggtccgatatcaccacgcccacgggtgtaccgggaaccgg  
aatcattgtctggcctgcgagctggtgccgaggaaactgtctctgaacagaagaaggaggacgtctctgactacgaga  
actcccagtacaaggagttcctagtcacctctcccaacgagaagctggccagaggtctgctcatgctggccgagctgt  
cttgcaagggtctcttgccactggcgagttactccaagcagaccattgagcttggccgatccgaccccgagtttggtg  
ttggcttcattgccagaaccgacctaaaggcgactctgaggactggcttattctgacccccggggtgggtcttgacg  
acaaggagagacgtctcggacagcagtaccgaactgttgaggatgtcatgtctaccggaacggatatcataattgtcg  
gccgaggtctgtacggccagaaccgagatcctattgaggaggccaagcgataaccagaaggctggctgggaggctt  
accagaagattaactgttagagggttagactatggatatgtaatttaactgtgtatatagagagcgtgcaagtatggagcg  
ctgttcagcttgatgatggtcagacgacctgtctgatcgagtatgtatggtactgcacaacctgtgtatccgcatggtct  
gtccaatggggcatgttgtgtgtttctcggaattcgtataacttcgtatagcaggagttatccgaagcgataattacctg  
ttatccctagcttatcgattcccacaagacgaacaagtgataggccgagagccgaggacgaggtggagtga

FIG. 46C

taattaccctgttatccctagcttatcgatagagaccgggttggcggcgtatttgtgtccaaaaaacagccccaattgcc  
ccaattgaccccaaatgacccagtagcgggcccaccccggcgagagcccccttcacccacatatcaaacctcccc  
cgggtccacacttgcggttaagggcgtagggtagctgcagctcgtgaatctacgcttgtcagactttgtactagttctttgt  
ctggccatccgggtaacccatgceggacgcaaaatagactactgaaaatttttgccttgggttggtggactttagccaag  
ggtataaaagaccaccgtccccgaattacctttctctctctctctctctctgtcaactcacaccgaaggatccatga  
agctttccaccatctcttcacagcctgcgtaccctggcgcgtaccaagcgaggctctcccaacccaccggagccg  
ccgctgtgaaggccgccttcagaccttggaaacgcctaccaccacttcgcttccccacgacgacctgcacccgt  
gttaactcgttcgacgacgagcgaaacggctggggctcttctgccatcgacggcctggacaccgccatctgatggg  
cgacgccgacatcgtgaacaccatctgcagtagctgccccagatcaacttcaccaccaccgccgtggccaaccagg  
gcctctctgtgttcgagaccaacatccgatacctggggcgccgtctgtctgcctacgacctgctgcgagggccctctct  
ctctggccaccaaccagacctgggtgaactctctgctgcgacaggcccagacctggccaacggcctgaagggtggt  
ttaccacccctctggcgtgcccagccccaccgtgttcttcaacccaccgtgcgacgatctggcgcctcttctaaca  
cgtggccgagatcggtctctgtgtgctggagtggaaccgactgtctgacctgaccggcaacccccagtagccccagc  
tgcccagaaggcgagcttacctgctgaacccaagggtctctccgaggcctggcccgactgatcggcaccttc  
gtgtctaccttcaacggcaacctccaggactcttcggctcttggctgacctgatggactctttctacgagtacctgatca  
agatgtacctgtacgaccccggtggccttcgccactacaaggaccgatgggtgctggccgcccactctaccatcgccc  
acctggcctctcaccctctacccgaaaggacctgaccttctgtctcttacaacggccagtctacctctccaaactctg  
gacacctggcttcttcgccggtggcaacttcacctggggcgccatcctgctgaacgagcagaagtacatcgacttcgg  
cataagctggcctcttctacttcgccacctacaaccagaccgctctggcatcgccccgagggcttcgctgggtg  
gactctgtaccggcgctggcggtctccccctctctcagctctggcttctactcttctgccggcttctgggtgaccgcc  
ccctactacatcctgcgacccgagacctggagctctgtactacgcctaccgagtaccggcgactctaagtggcag  
gacctggcctgggaggccttctctgccatcgaggacgcctgtcgagccgctctgcctactcttctataacgacgtga  
cccaggccaacgggtggcgagcctctgacgacatggagctttctggttcgccgaggccctgaagtacgectacctga  
tcttcgccgaggaatctgacgtgcaggtgcaggccaacggcggcaacaagttcgtgttcaacaccgagggccacccc  
ttctctatccgatcttctctcgacgagggcgccacctggcccatgacgagctgtaatagcctaggggtgtctgtggtatcta  
agctatttatcactcttflacaactctacctcaactatctactttaataaataaatatcgtttattctctatgattactgtatatgcgt  
tctctaaagacaaatcgaattccatgtgtaacactcgtctggagagttagtcacccgacagggttaactctaacttcccaa  
caccttattaactctgcgtaactgtaactcttcttgcacgtcgacttactcaatttctctcatctctgctggattgtgt  
ctatcgtctggctctaatacattattgtttattgcccacaacttcattgcacgtaagtgaattgtttataacagcgttcgc  
caattgctgcgccatcgtcgtccgctgtcctaccgttagggtagtgtgtctcacactaccgaggttactagagttgggaa  
agcgatactgcctcgg

FIG. 47A

acacaccacctggtcttacgactgcagagagaatcggegttacctcctcacaagccctcagtgcggccgccccgggt  
gggcgaagaactccagcatgagatccccgcgtggagatcatccagccggcgctccggaaaacgattccgaagcc  
caacctttcatagaaggcggcgggtggaatcgaaatctcgtgatggcaggttgggcgtcgttggctcggtcatttcgaacc  
ccagagtcccgtcagaagaactcgtcaagaaggcgatagaaggcgalgcgtgcgaatcgggagcggcgataccg  
taaagcacgaggaagcggtcagcccatcgccgccaagctcttcagcaatatcacgggtagccaacgctatgtcctgat  
agcggctccgcacaccagccggccacagtcgatgaatccagaaaagcggccattttccacatgatattcggcaagc  
aggcatcgccatgggtcacgacgagatcctcgccgtcgggcatgcgcgccttgagcctggcgaacagttcggctggc  
gcgagccctgatgctcttcgtccagatcatcctgatcgacaagaccggcttccatccgagtacgtgctcgcctgatgcg  
atgtttcgttgggtggtcgaatgggcaggtagccggatcaagcgtatgcagccgccgattgcatcagccatgatggata  
ctttctcggcaggagcaaggtagatgacaggagatcctgccccggcacttcgccaatagcagccagtcctttccgc  
ttcagtgacaacgtcgagcacagctgcgcaaggaaacgcccgtcgtggccagccacgatagccgcgtgcctcgtcctg  
cagttcattcagggcaccggacaggtcggctttgacaaaaagaaccgggcgccctgcgctgacagccggaacacgg  
cggcatcagagcagccgattgtctgttggccagtcatagccgaatagcctctccaccaagcggccgggagaacctgc  
gtgcaatccatcttgttcaatcatcgaaaacgacatcctcatcctgtctcttgatcagatcttgatccctgcgccatcagatcct  
tgggcggaagaaagccatccagtttactttgcagggttcccaacctaccagagggcgccccagctggcaattccgggt  
cgcttgcgtccataaaaccgcccagctagctatcgccatgtaagcccactgcaagctacctgctttctcttgcgcttgcg  
ttttcccttgccagatagccagtagctgacattcatccggggcagcaccgtttctgcggactggctttctacgtgttccg  
cttcttttagcagcccttgccctgagtgcttgcggcagcgtgaagctagcttatgcggtgtgaaataccgcacagatg  
cgtaaggagaaaataccgcatcaggcgtcttccgcttctcgtcactgactcgtgcgtcggctgttcggctgcggc  
gagcgggtatcagctcactcaaaggcggtaatacggttatccacagaatcaggggataacgcaggaaagaacatgtgag  
caaaaggccagcaaaaggccaggaaccgtaaaaggccgcttgcgtggcgtttttccataggctccgccccctgacg  
agcatcacaaaaatcgacgtcaagtcagaggtggcgaaacccgacaggactataaagataccaggcgtttccccctg  
gaagctccctcgtgcgtctcctgttccgacctgccgcttaccggatacctgtccgctttctccctcgggaagcgtgg  
cgctttctcatagctcacgtgtaggtatctcagttcgggtgtaggtcgttcgtccaagctgggctgtgtgcacgaaccccc  
cgttcagcccgaccgtgcgccttatccggtaactatcgtcttgagtccaacccggtaagacacgacttatcgccactgg  
cagcagccactggtaacaggattagcagagcgaggtatgtaggcgggtgctacagagttctgaagtgggtggcctaacta  
cggtacactagaaggacagtatitgggtatctgcgtctcgtgaagccagttaccttcggaaaaagagttggtagctcttg  
atccggcaaaacaaaccaccgctggtagcggcgggtttttgtttgcaagcagcagattacgcgcagaaaaaaaggatctc  
aagaagatcctttgatcttttttactgaacgggtatccccaccggaattgcggccgctgtcgggaaccgcgttcagggtg  
aacaggacacctcccttgcacttcttggtatatacagtaggctgatgtattcatagtgggggtttttcataataaatttact

FIG. 47B

aacggcaggcaacattcactcggcttaaacgcaaacggaccgtcttgatatcttctgacgcattgaccaccgaga  
aatagtgttagttaccgggtgagttattgttcttctacacaggcgacgcccacgtctagagttgatgtactaactcag  
attcactacctaccctatccctggtagcacaagcactttgctagatagagtcgacaaaggcgggccccccctc  
gagattaccctgttatecctacataacttcgtatagcatacattatacgaagttattctgaattccgagaaacacaaca  
catgccccattggacagaccatgcggatacacagggtgtgcagtaccatacatactcgatcagacaggctcgtctga  
ccatcatacaagctgaacagcgtccatacttgacgctctctatatacacagttaaattacatatccatagctaacct  
ctaacagttaatcttctggaagcctcccagccagccttctggtagcttggcctcctcaataggatctcggttctgg  
ccgtacagacctcggccgacaattatgatatccgttccggtagacatgacatcctcaacagttcggtagctgtctcc  
gagagcgtctcccttgctcgaagaccaccccggggggtcagaataagccagtcctcagagtcgcccttaggtcg  
gttctgggcaatgaagccaaccacaaaactcgggggtcggatcgggcaagctcaatggtctgcttggagtactcgcc  
agtggccagagagcccttgcaagacagctcggccagcatgagcagacctctggccagcttctcgttgggagagg  
ggactaggaactccttgtagtgggagttctcgtagtcagagacgtcctccttcttctgttcagagacagtttctcggc  
accagctcgcaggccagcaatgattccgggtccgggtacaccgtgggcgttggtgatatcggaacctcggcgat  
tcggtgacaccggtagtgggtgcttgacagtgttgccaatatctgcgaactttctgtcctcgaacaggaagaaaccgt  
gcttaagagcaagttccttgagggggagcacagtgcggcgtaggtgaagtcgtcaatgatgtcgatatgggtctt  
gatcatgcacacataagggtccgaccttaccgcaagctcaatgagctccttgggtgggtgtaacatccagagaagca  
cacagggttggttttcttggtgccacgagcttgagcactcgagcggcaaaggcggacttgtagcgttagctcgag  
cttcgtaggagggcattttggtggtgaagaggagactgaaataaatttagtctgcagaacttttatcggaaccttacc  
tggggcagtgaagtatatgttatggtaatagttacgagttagttgaacttatagatagactggactatacggctatcgg  
tccaaattagaaagaacgtcaatggctctctgggcgggaattcgtataacttcgtatagcaggagttatccgaagcga

FIG. 47C

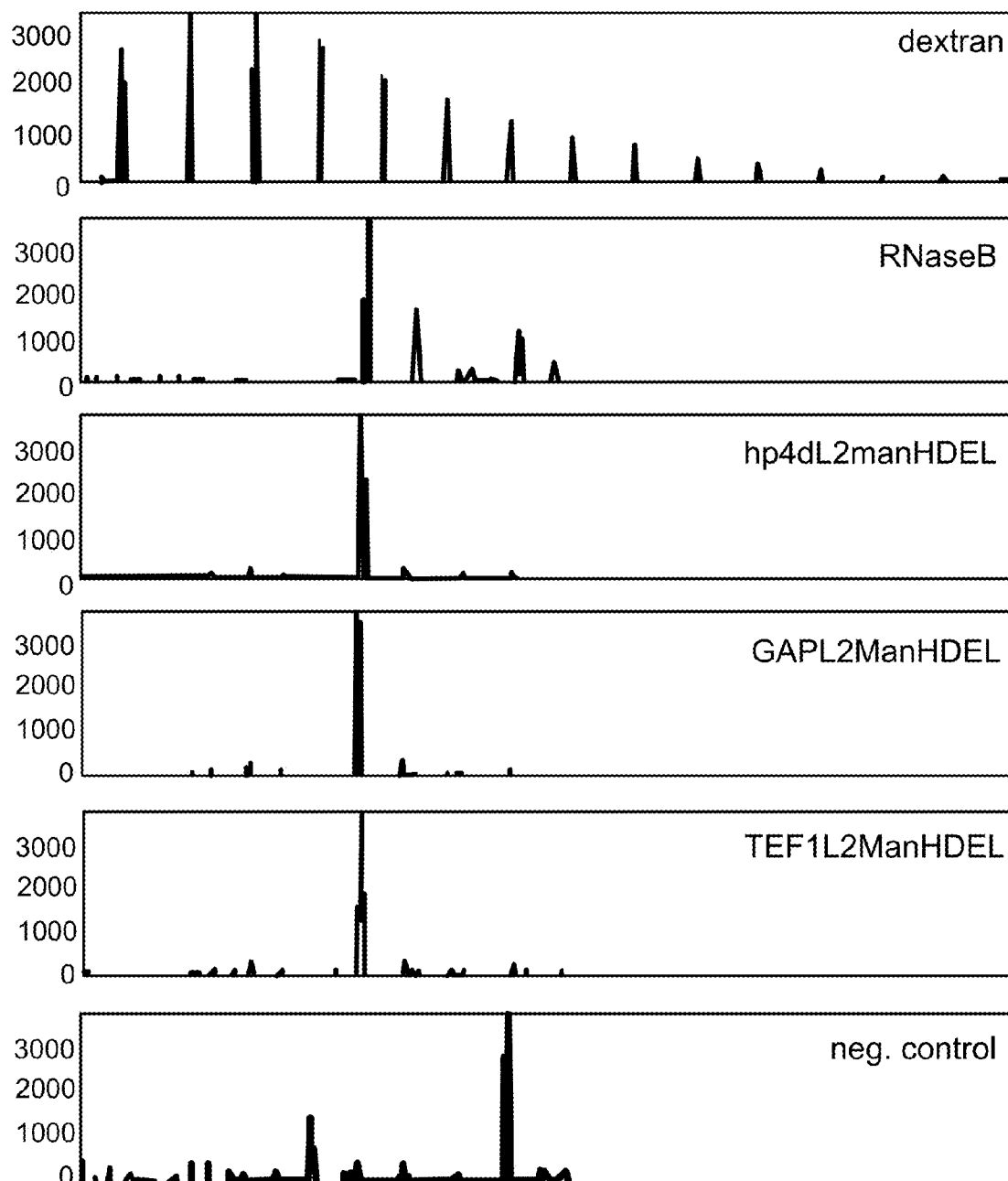


FIG. 48

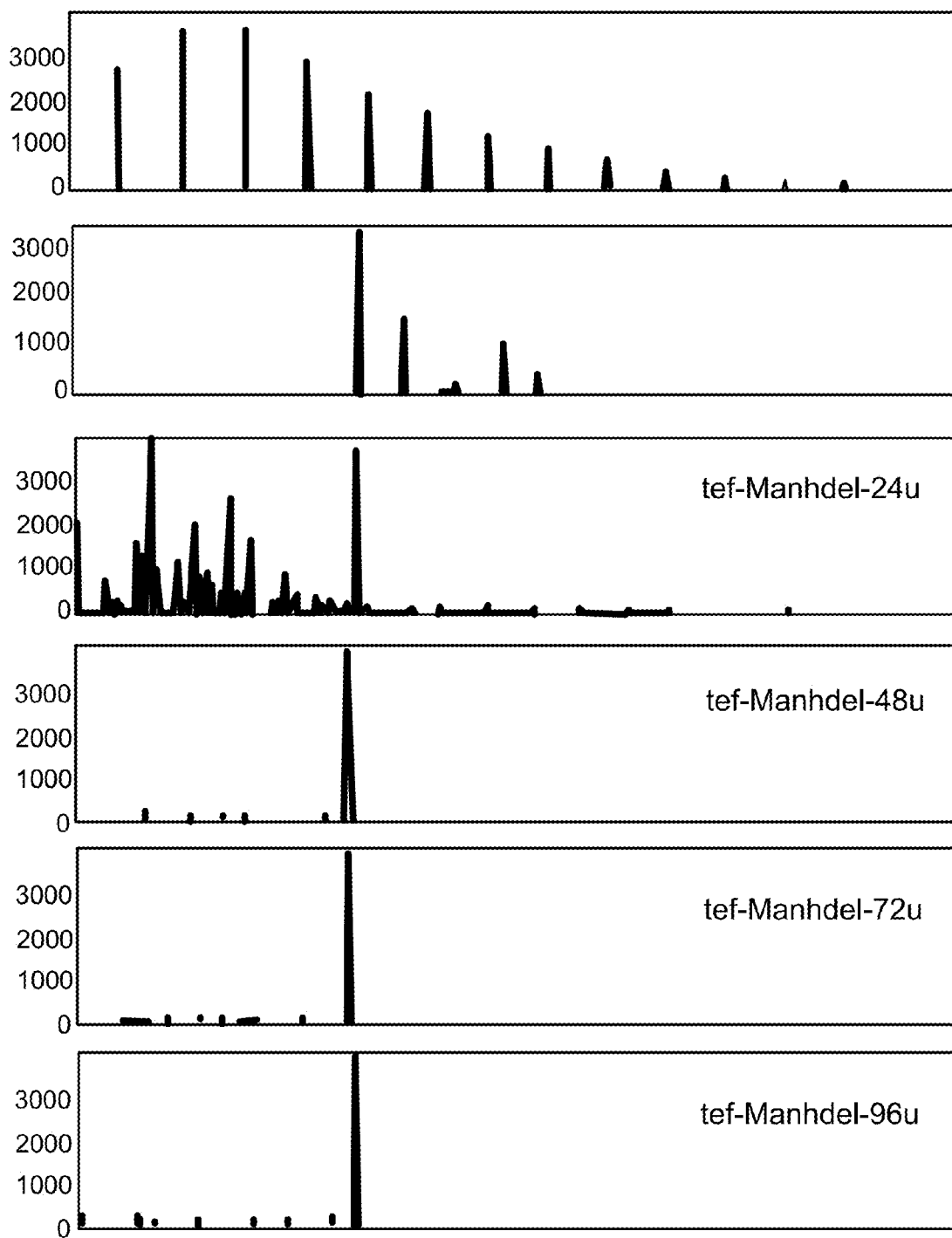


FIG. 49

gagctcagcgcctgaccctgtatccccaagtctttcggctactcttctgtggtgtgtgtgtgtaacgccacactgtgact  
cgftcgacccccccaccttccccgccctgggcaccttctctcgatacagctctacccgatctggccgacgaatggagct  
gtctatgggccccatccaggccaaccacaccggcaccggcctgctgctgacctgcagcccagcagaagtccaga  
aggctcaagggtctcggcgagccatgaccgacgccgctgccctgaacatcctggccctgtctcccccgctcagaac  
ctgctcctgaagtcttacttctctgaggagggcatcggctacaacatcatccgagtgcctatggcctcttctgacttctat  
ccgaacctacacctacgccgacacccccgacgacttcagctgcacaacttctccctgcccaggaggacaccaagct  
gaagateccccctgatccaccgagccctgcagctggcccagcgacccgtgtctctgtggccttccctggacctctcc  
acctggctcaagaccaacggcgccgtgaacggcaagggtctctgaagggccagcccgcgacatctaccaccaga  
cctgggcccgatacttctgaagtctctggacgcctacgccgagcacaagctgcagttctgggccgtgaccgccgaga  
acgagccctctgtggactgctgtccggctaccccttcagtgctgtgggttcacccccgagcaccagcgagacttcac  
gcccagagacctgggacccacctggccaactctaccaccacaacgtgcgactgctgatgctggacgaccagegact  
gctgctgccccactgggccaagggtggtgctgaccgacccccgaggccgccaagtacgtccacggcatcgtgtccatt  
ggatctggactttctggctcccgccaaagggccacctggggcgagacccaccgactgttcccaacaccatgctgttgc  
ctctgaggcttgtgtgggtcttaagttctgggagcagctctgtgcgactgggtcttgggaccgaggcatgcagtactctc  
actctatcatcaccacacctgtgtaccacgtgggtgggtggaccgactggaacctggccctgaaccccgaggcgggac  
ccaactgggtgcgaaactctgtgactctcccatcatcgtggacatcaccaaggacaccttctacaagcagcccatgttc  
taccacctggggccacttcttaagttcatccccgagggtctcagcgagtgggcctggtggcctctcagaagaacgacc  
tggacgccgtggccctgatgcaccccgacggctctgccgtggtggtggtcctgaaccgatcttctaaggacgtgccct  
gaccatcaaggacccccgccgtgggttctctggagaccatctctccggctactctatccacacctacgtggcgacga  
cagtaatagcctaggggtacc

FIG. 50

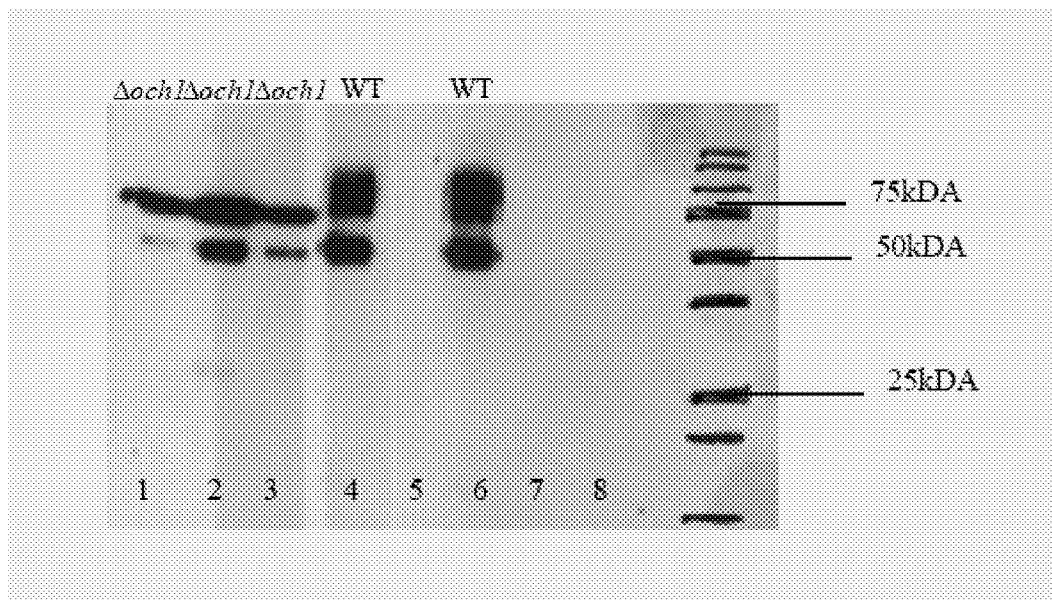


FIG. 51

gtcccccccgactgatctgtgactetgagtgtggaacgataacctgtggaagccaaggaagecgag  
aacatcaccacgggtgtgccgagcactgttctctgaacgagaacattacctgcecgacaccaagggtg  
aaattctacgcctggaagegaatggagggtgggccagcaggccgtggagggtgtggcagggactggctct  
gtgtctgagggcgtgtctgcgaggacaggctctcttggtgaactcttctcagecctgggagccccctgcag  
ctgcacgtggacaaggccgtgtctggcctgcgatctctgaccacctgtctgcgagccctcggtgtctcaga  
aggaagccatctctcccccgacgcgcctctgtgtccccctggaaccateaccgcecgacaccttcg  
aaagctgttccgagtgtactctaacttctgcgaggcaagctgaagctgtacaccggcgaggttgctg  
aaccggcgaccga

FIG. 52

ctggacaacggcctggccccgaacccccaccatgggctggctgcactgggagcgattcatgtgtaacct  
ggactgtcaggaagagccccgactcttgtatctctgagaagctgttcatggaaatggccgagctgatggt  
gtctgagggctggaaggacgccggctacgagtacctgtgtatcgacgactgttggatggccccccagc  
gagactctgagggccgactccaggccgacccccagcgattccccacggcatccgacagctcgcca  
actacgtgcactctaagggcctgaagctgggcatctacgccgacgtgggcaacaagacctgtgccgg  
cttccccggctctttcggctactacgacatcgacgccagaccttcgccgactggggcggtggacctgt  
gaagttcgacggctgttactgtgactctctcgagaacctggccgacggctacaagcacatgtctctggcc  
ctgaaccgaaccggccgatctatcgtgtactcttgtgagtggccccctgtacatgtggcccttcagaagc  
ccaactacaccgagatccgacagtactgtaaccactggcgaaacttcgccgacatcgacgactcgtgg  
aagtctatcaagtctattctggactggacctcttcaaccaggagcgaatcgtcgacgtcgccggacccg  
gcggatggaacgaccccgacatgctggtgatcggaacttcggcctgtcttggaaaccagcaggtgacc  
cagatggccctgtgggctatcatggctgccccctgttcatgtctaacgacctgcgacacatctctccc  
aggccaaggccctgtctcaggacaaggacgtgatcgccatcaaccaggacccccctgggcaagcagg  
gctaccagctccgacagggcgacaacttcgaggtgtgggagcgacccctgtctggcctggcctgggc  
cgtggccatgatcaaccgacaggagatcggeggaccccgatcttacaccatcgccgtggcctccctgg  
gaaagggcggtggcctgtaaccccgccctgttcatcaccagctcctgccgtgaagcgaagctggga  
ttctacgagtggacctctcgactcgatctcacatcaacccaccggcaccgtgctgctccagctcgag  
aacaccatgcagatgtctctgaaggacctgctg

FIG. 53

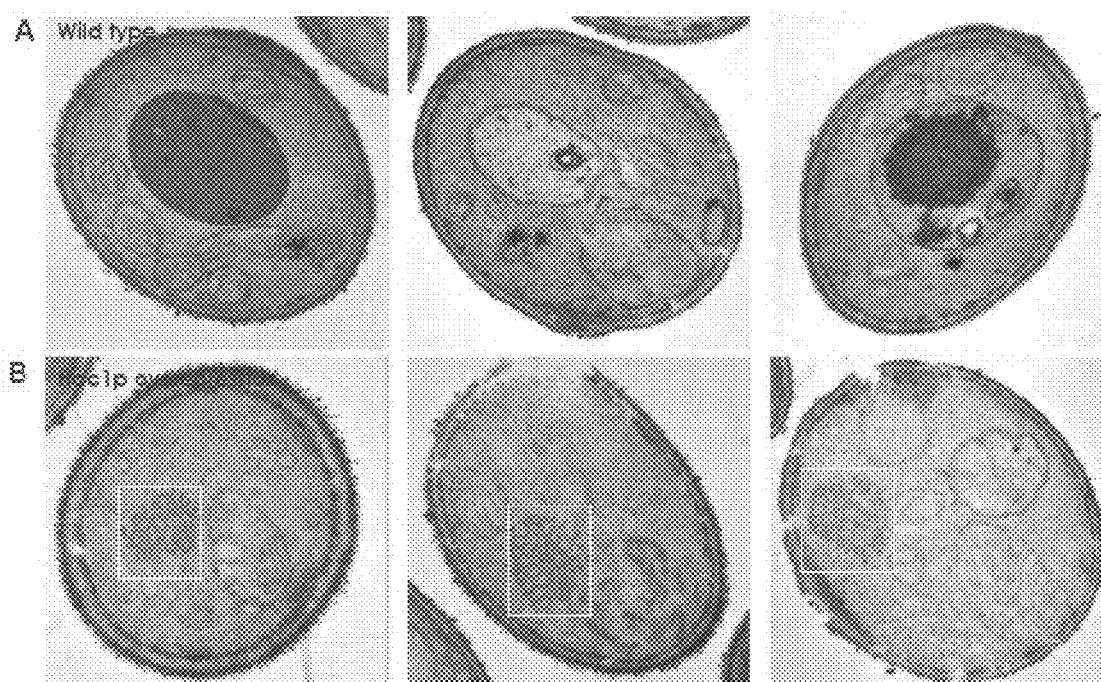


FIG. 54

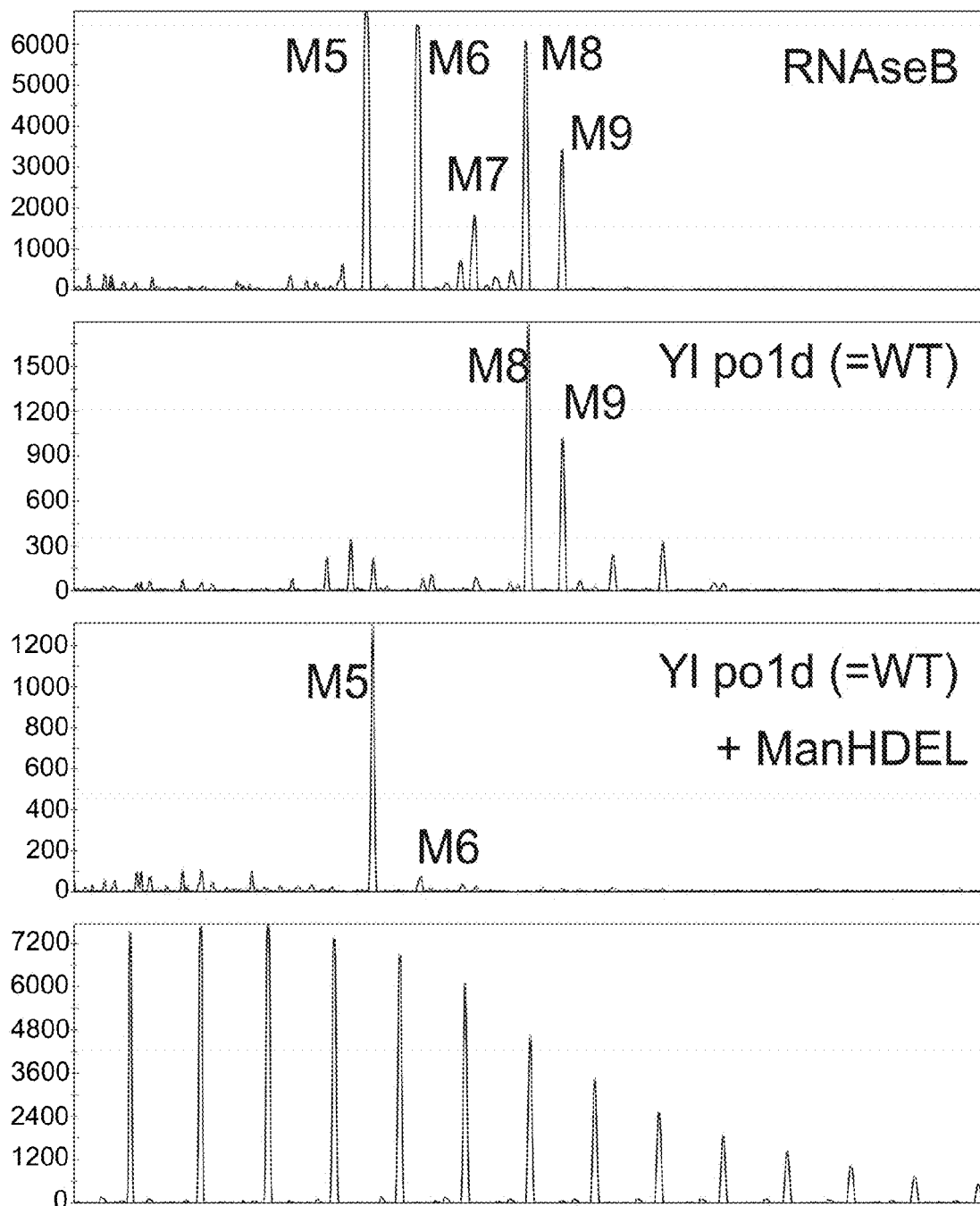


FIG. 55

1

# MICROORGANISMS GENETICALLY ENGINEERED TO HAVE MODIFIED N-GLYCOSYLATION ACTIVITY

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 13/095,532, filed Apr. 27, 2011, which is a continuation of U.S. application Ser. No. 12/062,469, filed Apr. 3, 2008, now U.S. Pat. No. 8,026,083, issued Sep. 27, 2011, which claims the benefit of U.S. Provisional Application Ser. No. 60/940,212, filed May 25, 2007, and which claims the benefit of U.S. Provisional Application Ser. No. 60/909,904, filed Apr. 3, 2007. The disclosures of all the prior applications are considered part of (and are incorporated by reference in) the disclosure of this application.

## TECHNICAL FIELD

The invention relates to methods of obtaining glycosylated molecules, particularly protein and lipid molecules.

## BACKGROUND

High performance expression systems are required to produce most biopharmaceuticals (e.g., recombinant proteins) currently under development. The biological activity of many of these biopharmaceuticals is dependent on their modification (e.g., phosphorylation or glycosylation). A yeast-based expression system combines the ease of genetic manipulation and fermentation of a microbial organism with the capability to secrete and to modify proteins. However, recombinant glycoproteins produced in yeast cells exhibit mainly heterogeneous high-mannose and hyper-mannose glycan structures, which can be detrimental to protein function, downstream processing, and subsequent therapeutic use, particularly where glycosylation plays a biologically significant role.

## SUMMARY

The present invention is based, at least in part, on: (a) the discovery that single gene deletion (Outer Chain elongation (OCH1) deletion) in *Yarrowia lipolytica* cells resulted in the substantially homogeneous production of glycosylated proteins having  $\alpha$ -1,2-linked mannose residues on a  $\text{Man}_5\text{GlcNAc}_2$  (structural formula IV; FIG. 1) backbone; (b) the discovery that overexpression of an engineered  $\alpha$ -1,2-mannosidase targeted to the ER of *Yarrowia lipolytica* cells (both with AND without OCH1 deletion) resulted in the substantially homogeneous production of glycosylated proteins carrying the  $\text{Man}_5\text{GlcNAc}_2$  N-glycan structure (structural formula IV; FIG. 1); (c) the discovery that inactivating the Asparagine Linked Glycosylation 3 (ALG3) enzyme activity in *Yarrowia lipolytica* cells results in highly increased levels of glucosylated glycans; and (d) the discovery that overexpression of a wild-type form of a *Yarrowia lipolytica* gene (MNN4) in *Yarrowia lipolytica* results in hyperphosphorylation of  $\alpha$ -1,2-linked mannose residues. Thus, the genetically engineered cells (e.g., *Yarrowia lipolytica*, *Arxula adeninivorans*, or other related species dimorphic yeast cells) can be used in methods to produce target molecules having an altered N-glycosylation form as compared to the N-glycosylation form of the target molecules produced in non-genetically engineered cells of the same species. As administration of N-glycosylated target molecules (e.g., N-glycosylated pro-

2

teins) to patients having a metabolic disorder (e.g., a lysosomal storage disorder) has been shown to ameliorate the symptoms of the disorder, the methods and cells described are useful for the preparation of N-glycosylated target molecules for the treatment of, inter alia, metabolic disorders such as lysosomal storage disorders.

The present invention is also based, at least in part, on the discovery of the spliced form of the *Yarrowia lipolytica* and *Pichia pastoris* HAC1 gene. The protein encoded by the HAC1 gene, Hac1p, is a transcriptional activator that activates transcription of several target genes by binding to a DNA sequence motif termed the Unfolded Protein Response (UPR) element. Among the Hac1p target genes are those that encode chaperones, foldases, and proteins which are responsible for lipid- and inositol metabolism. As the spliced form Hac1p is a more potent transcriptional activator than the form encoded by the unspliced HAC1 mRNA, overexpression of the spliced form of Hac1p transcription factor can lead to an increased expression of native and heterologous proteins as well as an increase in ER membrane. Thus, the spliced form of Hac1p can be used to increase the production of membrane and secreted proteins in a variety of eukaryotic cells (e.g., fungal cells (e.g., *Yarrowia lipolytica* or any other yeast cells described herein), plant cells, or animal cells (e.g., mammalian cells such as human cells) by simultaneous activation of the UPR and expression of target molecules.

The present invention is further based on the discovery of a mutant form of the MNS1 mannosidase capable of converting  $\text{Man}_8\text{GlcNAc}_2$  (structural formula I; FIG. 4) structures to  $\text{Man}_5\text{GlcNAc}_2$  (structural formula IV; FIG. 4),  $\text{Man}_6\text{GlcNAc}_2$  (structural formula V; FIG. 4) and  $\text{Man}_7\text{GlcNAc}_2$  (structural formula VI; FIG. 4) when expressed in *Yarrowia lipolytica*. Thus, genetically engineered eukaryotic cells (e.g., fungal cells (e.g., *Yarrowia lipolytica* or any other yeast cells described herein), plant cells, or animal cells (e.g., mammalian cells such as human cells)) expressing mutant forms of mannosidase such as MNS1 can be used in methods to produce target molecules having an altered N-glycosylation form as compared to the N-glycosylation form of the target molecules produced in non-genetically engineered cells of the same species. Therefore, the cells and methods described are useful for the preparation of N-glycosylated target molecules for the treatment of, inter alia, metabolic disorders such as lysosomal storage disorders (see below).

In one aspect, the disclosure features a method of producing an altered N-glycosylation form of a target protein. The method includes the step of introducing into a cell a nucleic acid encoding a target protein, wherein the cell produces the target protein in an altered N-glycosylation form and wherein the cell is a *Yarrowia lipolytica* or an *Arxula adeninivorans* cell (or a related species dimorphic yeast cell) genetically engineered to contain at least one modified N-glycosylation activity. The method can also include the step of providing the *Yarrowia lipolytica* or an *Arxula adeninivorans* cell (or related species dimorphic yeast cell) genetically engineered to contain at least one modified N-glycosylation activity. The method can also include the step of isolating the altered N-glycosylation form of the target protein.

In some embodiments, the target protein can be an endogenous protein or an exogenous protein. The target protein can be a mammalian protein such as a human protein. The target protein can be, for example, a pathogen protein, a lysosomal protein, a growth factor, a cytokine, a chemokine, an antibody or antigen-binding fragment thereof, or a fusion protein. The fusion protein can be, for example, a fusion of a pathogen protein, a lysosomal protein, a growth factor, a cytokine, or a

chemokine with an antibody or an antigen-binding fragment thereof. The target protein can be, for example, one associated with a lysosomal storage disorder (LSD). The target protein can be, for example, glucocerebrosidase, galactocerebrosidase, alpha-L-iduronidase, beta-D-galactosidase, beta-glucosidase, beta-hexosaminidase, beta-D-mannosidase, alpha-L-fucosidase, arylsulfatase B, arylsulfatase A, alpha-N-acetylglucosaminidase, aspartylglucosaminidase, iduronate-2-sulfatase, alpha-glucosaminidase-N-acetyltransferase, beta-D-glucuronidase, hyaluronidase, alpha-L-mannosidase, alpha-neuraminidase, phosphotransferase, acid lipase, acid ceramidase, sphingomyelinase, thioesterase, cathepsin K, or lipoprotein lipase.

In some embodiments, the altered N-glycosylation form can contain one or more N-glycan structures such as, e.g.,  $\text{Man}_5\text{GlcNAc}_2$ ,  $\text{Man}_8\text{GlcNAc}_2$ ,  $\text{Man}_9\text{GlcNAc}_2$ ,  $\text{Man}_3\text{GlcNAc}_2$ ,  $\text{Glc}_1\text{Man}_5\text{GlcNAc}_2$ ,  $\text{Glc}_2\text{Man}_5\text{GlcNAc}_2$ . In some embodiments, the altered glycosylation can be, for example,  $\text{Man}_5\text{GlcNAc}_2$ ,  $\text{Man}_8\text{GlcNAc}_2$ ,  $\text{Man}_9\text{GlcNAc}_2$ ,  $\text{Man}_3\text{GlcNAc}_2$ ,  $\text{Glc}_1\text{Man}_5\text{GlcNAc}_2$ ,  $\text{Glc}_2\text{Man}_5\text{GlcNAc}_2$ .

In some embodiments, the altered N-glycosylation form of the target protein can be homogenous or substantially homogenous. For example, the fraction of altered target molecules that contain the altered glycosylation can be at least about 20%, at least about 30%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95% or more.

In some embodiments, the cell can be genetically engineered to be deficient in at least one N-glycosylation activity. The N-glycosylation activity can be, for example, ALG3 activity, OCH<sub>1</sub> activity, MNS1 activity, or MNN9 activity.

In some embodiments, at least one modification can be: (a) deletion of a gene encoding a protein having the N-glycosylation activity; (b) expression of a mutant form of a protein having the N-glycosylation activity; (c) introduction or expression of an RNA molecule that interferes with the functional expression of a protein having the N-glycosylation activity; (d) expression of a protein having N-glycosylation activity (such as ALG6 or an alpha-mannosidase (e.g., an alpha-mannosidase targeted to the endoplasmic reticulum). The expressed protein can be a protein encoded by an exogenous nucleic acid in the cell. The expressed protein can be an alpha-mannosidase with a pH optimum below 7.5 (e.g., a pH optimum below 5.1). The protein having N-glycosylation activity can be an exogenous protein. The protein having N-glycosylation activity can be a mammalian protein (such as a human protein) or a lower eukaryotic (e.g., a fungus, a protozoan, or a trypanosome) protein. The lower eukaryote can be selected from the group consisting of *Typanosoma brucei*, *Trichoderma harzianum*, an *Aspergillus*, and any other lower eukaryote described herein.

In some embodiments, the N-glycosylation activity can be a glucosyltransferase activity. In some embodiments, the protein having N-glycosylation activity is ALG6 or an alpha-mannosidase. The alpha-mannosidase can be targeted to the endoplasmic reticulum. For example, the protein having N-glycosylation activity can be a fusion protein comprising an alpha-mannosidase polypeptide and an HDEL endoplasmic reticulum retention peptide.

In some embodiments, the protein having N-glycosylation activity can be a protein that is capable of removing glucose residues from  $\text{Man}_5\text{GlcNAc}_2$ . For example, the protein having N-glycosylation activity can be a protein having  $\alpha$ -1,3-

glucosidase activity such as, but not limited to, a glucosidase II (e.g., one or both of the alpha and beta subunit of a glucosidase II) or a mutanase.

In some embodiments, the cell can be genetically engineered to comprise at least two modified N-glycosylation activities such as any of the modified N-glycosylation activities described herein. The at least two modified N-glycosylation activities can comprise, e.g., a deficiency in an ALG3 activity and an elevated level of an ALG6 activity.

In some embodiments, the cell can be genetically engineered to comprise at least three modified N-glycosylation activities such as any of the modified N-glycosylation activities described herein. The at least three modified N-glycosylation activities can comprise, e.g., a deficiency in an ALG3 activity; an elevated level of an ALG6 activity; and an elevated level of a glucosidase II activity.

In some embodiments, the cell is not genetically engineered to be deficient in an OCH1 activity.

In some embodiments, modification can comprise expression of a protein or biologically active variant thereof capable of effecting mannosyl phosphorylation of the target protein. The protein or biologically active variant thereof capable of effecting mannosyl phosphorylation can be MNN4, PNO1, or MNN6. In some embodiments, at least about 30% of the mannosyl residues of a glycoprotein can be phosphorylated.

In some embodiments, the method can further include additional processing of the glycoprotein. The additional processing can occur in vitro or in vivo. The additional processing can comprise addition of a heterologous moiety to the modified glycoprotein. The heterologous moiety can be a polymer or a carrier. The additional processing can comprise enzymatic or chemical treatment of the altered N-glycosylation form of the target protein. For example, the additional processing can comprise treatment of the altered N-glycosylation form of the target protein with a mannosidase, a mannanase, a phosphodiesterase, a glucosidase, or a glucosyltransferase. The additional processing can include treatment of the altered N-glycosylation form of the target protein with hydrofluoric acid. The additional processing can include phosphorylation of the altered N-glycosylation form of the target protein.

In another aspect, the disclosure provides a method of producing an altered N-glycosylation form of a target protein. The method includes the steps of: providing a eukaryotic cell (e.g., a fungal cell, a plant cell, or an animal cell) genetically engineered to comprise at least one modified N-glycosylation activity; and introducing into the cell a nucleic acid encoding a target protein, wherein the cell produces the target protein in an altered N-glycosylation form.

In another aspect, the disclosure features a method of producing an altered N-glycosylation form of a target protein. The method includes the step of contacting a target protein with a cell lysate prepared from a *Yarrowia lipolytica* or an *Arxula adeninivorans* cell genetically engineered to comprise at least one modified N-glycosylation activity, wherein the contacting of the target protein with the cell lysate results in an altered N-glycosylation form of the target protein.

In yet another aspect, the disclosure features a method of producing an altered N-glycosylation form of a target protein, which method includes the step of contacting a target protein with one or more proteins having N-glycosylation activity, wherein the one or more proteins having N-glycosylation activity are obtained from a *Yarrowia lipolytica* or an *Arxula adeninivorans* cell genetically engineered to comprise at least one modified N-glycosylation activity and wherein contact-

5

ing the target molecule with the one or more proteins having N-glycosylation activity results in an altered N-glycosylation form of the target protein.

In another aspect, the disclosure provides an isolated protein having altered N-glycosylation, wherein the protein is produced by any of the methods described above.

In yet another aspect, the disclosure provides an isolated *Yarrowia lipolytica* or *Arxula adeninivorans* cell (or other related species dimorphic yeast cell) genetically engineered to comprise at least one modified N-glycosylation activity. The N-glycosylation activity can be, for example, ALG3 activity, OCH1 activity, MNS1 activity, or MNN9 activity. The modification can be any of those described herein. For example, the modification can include: (a) deletion of a gene encoding a protein having the N-glycosylation activity, (b) expression of a mutant form of a protein having the N-glycosylation activity, (c) introduction or expression of an RNA molecule that interferes with the functional expression of a protein having the N-glycosylation activity, or (d) expression of a protein having N-glycosylation activity. The protein having N-glycosylation activity can be, for example, ALG6. The protein having N-glycosylation activity can be a mammalian protein such as a human protein. The modification can also include expression of a protein (e.g., MNN4 or PNO1) or biologically active variant thereof capable of promoting mannosyl phosphorylation of the modified glycoprotein.

In another aspect, the disclosure provides a method of treating a disorder treatable by administration of a protein having altered N-glycosylation. The method includes the steps of administering to a subject a protein obtained by any of the methods described above, wherein the subject is one having, or suspected of having, a disease treatable by administration of a protein having altered N-glycosylation. The method can also include the steps of (a) providing a subject and/or (b) determining whether the subject has a disease treatable by administration of a protein having altered N-glycosylation. The subject can be mammal such as a human. The disorder can be, for example, a cancer, an immunological disorder (e.g., an inflammatory condition) or a metabolic disorder. The metabolic disorder can be any of those described herein, e.g., a lysosomal storage disorder (LSD) such as Gaucher disease, Tay-Sachs disease, Pompe disease, Niemann-Pick disease, or Fabry disease. The protein can be one associated with an LSD, e.g., the protein can be, for example, glucocerebrosidase, alpha-galactosidase. The protein can be, for example, alpha-L-iduronidase, beta-D-galactosidase, beta-glucosidase, beta-hexosaminidase, beta-D-mannosidase, alpha-L-fucosidase, arylsulfatase B, arylsulfatase A, alpha-N-acetylgalactosaminidase, aspartylglucosaminidase, iduronate-2-sulfatase, alpha-glucosaminide-N-acetyltransferase, beta-D-glucuronidase, hyaluronidase, alpha-L-mannosidase, alpha-neuromimidine, phosphotransferase, acid lipase, acid ceramidase, sphingomyelinase, thioesterase, cathepsin K, or lipoprotein lipase.

In another aspect, the disclosure provides a substantially pure culture of *Yarrowia lipolytica* or *Arxula adeninivorans* cells (or other related species dimorphic yeast cells), a substantial number of which being genetically engineered to comprise at least one modified N-glycosylation activity (such as any of the modifications described herein). The culture of cells can contain one or more subpopulations of cells, each subpopulation comprising a different modified glycosylation activity.

In yet another aspect, the disclosure provides: (a) an isolated nucleotide sequence comprising SEQ ID NO:1 or SEQ ID NO:2; (b) an isolated nucleotide sequence comprising a sequence that is at least 80% identical to SEQ ID NO:1 or

6

SEQ ID NO:2; or (c) a polypeptide encoded by the isolated nucleotide sequence of (a) or (b). In some embodiments, the isolated nucleic acid sequence is SEQ ID NO:1 or SEQ ID NO:2.

In another aspect, the disclosure features an isolated nucleic acid containing: (a) a nucleotide sequence that hybridizes under highly stringent conditions to the complement of SEQ ID NO:1 or SEQ ID NO:2; or (b) the complement of the nucleotide sequence.

In yet another aspect, the disclosure provides: (a) an isolated nucleotide sequence comprising (or consisting of) any of the nucleic acid sequences depicted herein; (b) an isolated nucleotide sequence comprising a sequence that is at least 80% identical to any of the nucleic acid sequences depicted herein; or (c) a polypeptide encoded by the isolated nucleotide sequence of (a) or (b). In some embodiments, the isolated nucleic acid sequence is any of the nucleic acid sequences depicted herein.

In another aspect, the disclosure features an isolated nucleic acid containing: (a) a nucleotide sequence that hybridizes under highly stringent conditions to the complement of any of the nucleic acid sequences depicted herein; or (b) the complement of the nucleotide sequence.

In yet another aspect, the disclosure provides: (a) a vector comprising any of the nucleic acid sequences described above or (b) a cultured cell containing the vector of (a). The vector can be an expression vector. The nucleic acid sequence in the vector can be operably linked to expression control sequence.

In another aspect, the disclosure provides a method for producing a protein. The method includes the step of culturing any of the cells described above under conditions permitting the expression of the polypeptide. The method can also include the step of, after culturing the cell, isolating the polypeptide from the cell or the medium in which the cell was cultured. The cell can be, e.g., a cultured cell containing a vector comprising any of the nucleic acid sequences described above.

The target molecules (e.g., target proteins), proteins having N-glycosylation activity, and altered N-glycosylation molecules described herein (collectively referred to as "molecules of the invention") can, but need not, be isolated. The term "isolated" as applied to any of the molecules of the invention described herein refers to a molecule, or a fragment thereof, that has been separated or purified from components (e.g., proteins or other naturally-occurring biological or organic molecules) which naturally accompany it. It is understood that recombinant molecules (e.g., recombinant proteins) will always be "isolated." Typically, a molecule of the invention is isolated when it constitutes at least 60%, by weight, of the total molecules of the same type in a preparation, e.g., 60% of the total molecules of the same type in a sample. For example, an altered glycosylation protein is isolated when it constitutes at least 60%, by weight, of the total protein in a preparation or sample. In some embodiments, a molecule of the invention in the preparation consists of at least 75%, at least 90%, or at least 99%, by weight, of the total molecules of the same type in a preparation.

As used herein, an "altered N-glycosylation form" of a target molecule is an N-glycosylation form of a target molecule produced by a genetically engineered host cell (e.g., *Yarrowia lipolytica* cell, *Arxula adeninivorans* cell, or a cell of another related dimorphic yeast cell species) that differs from the N-glycosylation form of the target molecule produced in a non-genetically engineered cell of the same species as the genetically engineered cell. Thus, an altered glycosylation form of a target molecule can be, for example, a form of the target molecule that is not N-glycosylated. More-

over, an altered glycosylation form of a target molecule can be, e.g., a form of the target molecule that has altered phosphorylation of one or more N-linked glycans.

As used herein, the term "other related dimorphic yeast cell species" refers to yeasts related to *Yarrowia lipolytica* and *Arxula adeninivorans* that belong to the family Dipodascaceae such as *Arxula*, *Dipodascus* (e.g. *D. albidus*, *D. ingens*, or *D. specifer*), *Galactomyces* (e.g. *G. reesii* or *G. geotrichum*), *Sporopachyderma*, *Stephanoascus* (e.g., *S. ciferii*), *Wickerhamiella*, and *Zygoascus*. Specifically, yeasts in the clade *Metchnikowia* (e.g., *M. pulcherrima* or *M. agaves*) and *Stephanoascus* (to which *Y. lipolytica* is assigned by analysis of the D1/D2 domain of the 26S-rDNA sequences of species such as *Arxula* (e.g. *A. adeninivorans* or *A. terrestris*)) and some *Candida* species (e.g., *C. apicola* but not *C. albicans*, *C. maltosa*, or *C. tropicalis*).

"Polypeptide" and "protein" are used interchangeably and mean any peptide-linked chain of amino acids, regardless of length or post-translational modification.

The disclosure also provides (i) biologically active variants and (ii) biologically active fragments or biologically active variants thereof, of the wild-type, full-length, mature "target proteins" or "proteins having N-glycosylation activity" described herein. Biologically active variants of full-length, mature, wild-type proteins or fragments of the proteins can contain additions, deletions, or substitutions. Proteins with substitutions will generally have not more than 50 (e.g., not more than one, two, three, four, five, six, seven, eight, nine, ten, 12, 15, 20, 25, 30, 35, 40, or 50) conservative amino acid substitutions. A conservative substitution is the substitution of one amino acid for another with similar characteristics. Conservative substitutions include substitutions within the following groups: valine, alanine and glycine; leucine, valine, and isoleucine; aspartic acid and glutamic acid; asparagine and glutamine; serine, cysteine, and threonine; lysine and arginine; and phenylalanine and tyrosine. The non-polar hydrophobic amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Any substitution of one member of the above-mentioned polar, basic or acidic groups by another member of the same group can be deemed a conservative substitution. By contrast, a non-conservative substitution is a substitution of one amino acid for another with dissimilar characteristics.

Deletion variants can lack one, two, three, four, five, six, seven, eight, nine, ten, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid segments (of two or more amino acids) or non-contiguous single amino acids.

Additions (addition variants) include fusion proteins containing: (a) full-length, wild-type, mature polypeptides or fragments thereof containing at least five amino acids; and (b) internal or terminal (C or N) irrelevant or heterologous amino acid sequences. In the context of such fusion proteins, the term "heterologous amino acid sequences" refers to an amino acid sequence other than (a). A fusion protein containing a peptide described herein and a heterologous amino acid sequence thus does not correspond in sequence to all or part of a naturally occurring protein. A heterologous sequence can be, for example a sequence used for purification of the recombinant protein (e.g., FLAG, polyhistidine (e.g., hexahistidine), hemagglutinin (HA), glutathione-S-transferase (GST), or maltose-binding protein (MBP)). Heterologous sequences can also be proteins useful as diagnostic or detectable mark-

ers, for example, luciferase, green fluorescent protein (GFP), or chloramphenicol acetyl transferase (CAT). In some embodiments, the fusion protein contains a signal sequence from another protein. In certain host cells (e.g., yeast host cells), expression and/or secretion of the target protein can be increased through use of a heterologous signal sequence. In some embodiments, the fusion protein can contain a carrier (e.g., KLH) useful, e.g., in eliciting an immune response (e.g., for antibody generation; see below) or endoplasmic reticulum or Golgi apparatus retention signals. Heterologous sequences can be of varying length and in some cases can be a longer sequences than the full-length target proteins to which the heterologous sequences are attached.

A "fragment" as used herein, refers to a segment of the polypeptide that is shorter than a full-length, immature protein. Fragments of a protein can have terminal (carboxy or amino-terminal) and/or internal deletions. Generally, fragments of a protein will be at least four (e.g., at least five, at least six, at least seven, at least eight, at least nine, at least 10, at least 12, at least 15, at least 18, at least 25, at least 30, at least 35, at least 40, at least 50, at least 60, at least 65, at least 70, at least 75, at least 80, at least 85, at least 90, or at least 100 or more) amino acids in length.

Biologically active fragments or biologically active variants of the target proteins or proteins having N-glycosylation activity have at least 25% (e.g., at least: 30%; 40%; 50%; 60%; 70%; 75%; 80%; 85%; 90%; 95%; 97%; 98%; 99%; 99.5%, or 100% or even greater) of the activity of the wild-type, full-length, mature protein. In the case of a target protein, the relevant activity is the ability of the target protein to undergo altered N-glycosylation in a genetically engineered cell. In the case of a protein having N-glycosylation activity, the relevant activity is N-glycosylation activity.

Depending on their intended use, the proteins, biologically active fragments, or biologically active variants thereof can be of any species, such as, e.g., fungus (including yeast), nematode, insect, plant, bird, reptile, or mammal (e.g., a mouse, rat, rabbit, hamster, gerbil, dog, cat, goat, pig, cow, horse, whale, monkey, or human). In some embodiments, biologically active fragments or biologically active variants include immunogenic and antigenic fragments of the proteins. An immunogenic fragment is one that has at least 25% (e.g., at least: 30%; 40%; 50%; 60%; 70%; 75%; 80%; 85%; 90%; 95%; 97%; 98%; 99%; 99.5%, or 100% or even more) of the ability of the relevant full-length, immature protein to stimulate an immune response (e.g., an antibody response or a cellular immune response) in an animal of interest. An antigenic fragment of a protein is one having at least 25% (e.g., at least: 30%; 40%; 50%; 60%; 70%; 75%; 80%; 85%; 90%; 95%; 97%; 98%; 99%; 99.5%, or 100% or even greater) of the ability of the relevant full-length, immature protein to be recognized by an antibody specific for the protein or a T cell specific to the protein.

"N-glycosylation activity" as used herein refers to any activity that is (i) capable of adding N-linked glycans to a target molecule (i.e., an oligosaccharyltransferase activity); (ii) removing N-linked glycans from a target molecule, (iii) modifying one or more N-linked glycans on a target molecule, (iv) modifying dolichol-linked oligosaccharides; or (v) is capable of aiding the activity of the activities under (i-iv). As such, N-glycosylation activity includes, e.g., N-glycosidase activity, glycosidase activity, glycosyltransferase activity, sugar nucleotide synthesis, modification, or transporter activity. Modification of one or more N-linked glycans on a target molecule includes the action of a mannosylphosphoryltransferase activity, a kinase activity, or a phosphatase activity, e.g., a mannosylphosphoryltransferase, a kinase, or a

phosphatase activity that alters the phosphorylation state of N-linked glycans on target molecules.

As used herein, to “genetically engineer” a cell or a “genetically engineered cell” and like terminology refers to any artificially created genetic alteration of a cell that results in at least one modified N-glycosylation activity in the cell as compared to a non-genetically engineered cell (e.g., a fungal cell such as *Yarrowia lipolytica* cell, *Arxula adeninivorans* cell, or other related species dimorphic yeast cell, a plant cell, or an animal cell (e.g., a mammalian cell such as a human cell)). Thus, it is understood that artificially created genetic alterations do not include, e.g., spontaneous mutations. Examples of artificial genetic alterations are described below (see “Genetically Engineered Cells”).

As used herein, the term “wild-type” as applied to a nucleic acid or polypeptide refers to a nucleic acid or a polypeptide that occurs in, or is produced by, respectively, a biological organism as that biological organism exists in nature.

The term “heterologous” as applied herein to a nucleic acid in a host cell or a polypeptide produced by a host cell refers to any nucleic acid or polypeptide (e.g., an protein having N-glycosylation activity) that is not derived from a cell of the same species as the host cell. Accordingly, as used herein, “homologous” nucleic acids, or proteins, are those that occur in, or are produced by, a cell of the same species as the host cell.

The term “exogenous” as used herein with reference to nucleic acid and a particular host cell refers to any nucleic acid that does not occur in (and cannot be obtained from) that particular cell as found in nature. Thus, a non-naturally-occurring nucleic acid is considered to be exogenous to a host cell once introduced into the host cell. It is important to note that non-naturally-occurring nucleic acids can contain nucleic acid subsequences or fragments of nucleic acid sequences that are found in nature provided that the nucleic acid as a whole does not exist in nature. For example, a nucleic acid molecule containing a genomic DNA sequence within an expression vector is non-naturally-occurring nucleic acid, and thus is exogenous to a host cell once introduced into the host cell, since that nucleic acid molecule as a whole (genomic DNA plus vector DNA) does not exist in nature. Thus, any vector, autonomously replicating plasmid, or virus (e.g., retrovirus, adenovirus, or herpes virus) that as a whole does not exist in nature is considered to be non-naturally-occurring nucleic acid. It follows that genomic DNA fragments produced by PCR or restriction endonuclease treatment as well as cDNAs are considered to be non-naturally-occurring nucleic acid since they exist as separate molecules not found in nature. It also follows that any nucleic acid containing a promoter sequence and polypeptide-encoding sequence (e.g., cDNA or genomic DNA) in an arrangement not found in nature is non-naturally-occurring nucleic acid. A nucleic acid that is naturally-occurring can be exogenous to a particular cell. For example, an entire chromosome isolated from a cell of yeast x is an exogenous nucleic acid with respect to a cell of yeast y once that chromosome is introduced into a cell of yeast y.

It will be clear from the above that “exogenous” nucleic acids can be “homologous” or “heterologous” nucleic acids. In contrast, the term “endogenous” as used herein with reference to nucleic acids or genes (or proteins encoded by the nucleic acids or genes) and a particular cell refers to any nucleic acid or gene that does occur in (and can be obtained from) that particular cell as found in nature.

As an illustration of the above concepts, an expression plasmid encoding a *Y. lipolytica* ALG6 protein that is transformed into a *Y. lipolytica* cell is, with respect to that cell, an

exogenous nucleic acid. However, the ALG6 protein coding sequence and the ALG6 protein produced by it are homologous with respect to the cell. Similarly, an expression plasmid encoding a *Arxula adeninivorans* ALG6 protein that is transformed into a *Y. lipolytica* cell is, with respect to that cell, an exogenous nucleic acid. In contrast with the previous example, however, the ALG6 protein coding sequence and the ALG6 protein produced by it are heterologous with respect to the cell.

As used herein, a “promoter” refers to a DNA sequence that enables a gene to be transcribed. The promoter is recognized by RNA polymerase, which then initiates transcription. Thus, a promoter contains a DNA sequence that is either bound directly by, or is involved in the recruitment, of RNA polymerase. A promoter sequence can also include “enhancer regions,” which are one or more regions of DNA that can be bound with proteins (namely, the trans-acting factors, much like a set of transcription factors) to enhance transcription levels of genes (hence the name) in a gene-cluster. The enhancer, while typically at the 5' end of a coding region, can also be separate from a promoter sequence and can be, e.g., within an intronic region of a gene or 3' to the coding region of the gene.

As used herein, “operably linked” means incorporated into a genetic construct so that expression control sequences effectively control expression of a coding sequence of interest.

Variants of any of the nucleic acid sequences described herein (e.g., the HAC1 sequences as depicted in SEQ ID NO:1 or SEQ ID NO:2) can have a sequence that is homologous, e.g., a sequence bearing at least about 70% (e.g., at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99%) homologous (identical) to the wild-type nucleic acid sequence. Such wild-type sequences can be isolated from nature or can be produced by recombinant or synthetic methods. Thus a wild-type sequence nucleic acid can have the nucleic acid sequence of naturally occurring human nucleic acid sequences, monkey nucleic acid sequences, murine nucleic acid sequences, or any other species that contains a homologue of the wild-type nucleic acid of interest. As used herein, a “homologous” or “homologous nucleic acid sequence” or similar term, refers to sequences characterized by homology at the nucleotide level of at least a specified percentage and is used interchangeably with sequence identity.

Percent homology or identity can be determined by, for example, the Gap program (Wisconsin Sequence Analysis Package, Version 8 for UNIX, Genetics Computer Group, University Research Park, Madison, Wis.), using default settings, which uses the algorithm of Smith and Waterman ((1981) Adv. Appl. Math. 2:482-489). In some embodiments, homology between a probe and target (see below) is between about 50% to about 60%. In some embodiments, homology between a probe and target nucleic acid is between about 55% to 65%, between about 65% to 75%, between about 70% to 80%, between about 75% and 85%, between about 80% and 90%, between about 85% and 95%, or between about 90% and 100%.

The term “probe,” as used herein, refers to nucleic acid sequences of variable length. In some embodiments, probes comprise at least 10 and as many as 6,000 nucleotides. In some embodiments probes comprise at least 12, at least 14, at least 16, at least 18, at least 20, at least 25, at least 50 or at least 75 or 100 contiguous nucleotides. Longer length probes are usually obtained from natural or recombinant sources (as opposed to direct, chemical synthesis), are highly specific to the target sequence, and are much slower to hybridize to the

target than longer oligomers. Probes can be single or double stranded nucleic acid molecules.

In some embodiments, a variant nucleic acid described herein can have a sequence comprising one or both strands with partial complementary (e.g., at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% complementary) to a region, portion, domain, or segment of the wild-type nucleic acid of interest (e.g., the HAC1 nucleic acid sequences as depicted in SEQ ID NO:1 or SEQ ID NO:2). In some embodiments, a variant nucleic acid sequence of interest can have a sequence comprising one or both strands with full complementary (i.e., 100% complementary) to a region, portion, domain, or segment of the wild-type nucleic acid sequence. Sequence "complementarity" refers to the chemical affinity between specific nitrogenous bases as a result of their hydrogen bonding properties (i.e., the property of two nucleic acid chains having base sequences such that an antiparallel duplex can form where the adenines and uracils (or thymine, in the case of DNA or modified RNA) are apposed to each other, and the guanines and cytosines are apposed to each other). Fully complementary sequences, thus, would be two sequences that have complete one-to-one correspondence (i.e., adenine to uracil/thymidine and guanine to cytosine) of the base sequences when the nucleotide sequences form an antiparallel duplex.

Hybridization can also be used as a measure of homology between two nucleic acid sequences. A nucleic acid sequence described herein, or a fragment or variant thereof, can be used as a hybridization probe according to standard hybridization techniques. The hybridization of a certain probe of interest (e.g., a probe of a HAC1 nucleotide sequence, e.g., the HAC1 nucleotide sequences as depicted in SEQ ID NOS:1 or 2) to DNA or RNA from a test source (e.g., a eukaryotic cell) is an indication of the presence of DNA or RNA (e.g., a HAC1 nucleotide sequence) corresponding to the probe in the test source. Hybridization conditions are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., 6.3.1-6.3.6, 1991. Moderate hybridization conditions are defined as equivalent to hybridization in 2× sodium chloride/sodium citrate (SSC) at 30° C., followed by a wash in 1×SSC, 0.1% SDS at 50° C. Highly stringent conditions are defined as equivalent to hybridization in 6× sodium chloride/sodium citrate (SSC) at 45° C., followed by a wash in 0.2×SSC, 0.1% SDS at 65° C.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the exemplary methods and materials are described below. All publications, patent applications, patents, Genbank® Accession Nos, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present application, including definitions, will control. The materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention, e.g., methods of producing altered N-glycosylation molecules, will be apparent from the following detailed description, and from the claims.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram depicting N-glycan precursor synthesis at the yeast endoplasmic reticulum. Genes whose encoded protein has an activity mediating the indi-

cated enzymatic conversions are in shaded boxes (e.g., ALG7; upper left). "UDP" and "UMP" refer to uridine diphosphate and uridine monophosphate, respectively. "GDP" and "GMP" refer to guanosine diphosphate and guanosine monophosphate respectively. "Gn" refers to N-acetylglucosamine. "M" refers to monomeric mannose, G refers to glucose, Pi refers to phosphate

FIG. 2 is a schematic diagram depicting N-glycan processing in the yeast endoplasmic reticulum.

FIG. 3 is a schematic diagram depicting N-glycan processing in the *S. cerevisiae* Golgi apparatus. Genes whose encoded protein has an activity mediating the indicated enzymatic conversions are in shaded boxes (e.g., OCH1; middle left).

FIGS. 4A and 4B are a schematic diagrams depicting the structure of the various N-glycan structures described herein.

FIGS. 5A-5E are schematic diagrams depicting the cloning strategy for OCH1 gene disruption in *Yarrowia lipolytica*. "PCR" refers to polymerase chain reaction.

FIGS. 6A and 6B are schematic diagrams depicting the cloning strategy for MNN9 gene disruption fragment. "PCR" refers to polymerase chain reaction.

FIG. 7 is a series of electroferograms depicting N-glycan analysis of mannoproteins obtained from wild-type *Yarrowia lipolytica* cells or glycosylation mutant (e.g., *Doch1 c19*, *Δmnn9 1* and *Δoch1 Δmnn9*) cells and MTLY60 strain cells. In some cases, the N-glycans were further treated with  $\alpha$ -1,2 mannosidase. Analysis was performed using DNA sequencer-assisted, fluorophore-assisted carbohydrate electrophoresis (DSA-FACE). "M5," "M6," "M7," "M8," and "M9," refer to the number of mannose residues conjugated to the base N-acetylglucosamine structure. The Y-axis represents the relative fluorescence units as an indication of the amount of each of the mannose structures. The X-axis represents the relative mobility of each complex mannose structure through a gel. The top electroferogram is an analysis of oligomaltose for use as a mobility standard.

FIGS. 8A-8E are schematic diagrams depicting the cloning strategy for *S. cerevisiae* MNS1 expression vector. "PCR" refers to polymerase chain reaction.

FIG. 9 is a series of electroferograms depicting N-glycan analysis of secreted glycoproteins obtained from MTLY60 cells expressing wild-type (WT) Mns1p or various mutant forms (i.e., R273G, R273L, or R269S/S272G/R273L) of Mns1p as indicated. Analysis was performed using DSA-FACE. "M5," "M6," "M7," "M8," "M9," refers to the number of mannose residues conjugated to the base N-acetylglucosamine structure. The Y-axis represents the relative fluorescence units as an indication of the amount of each of the mannose structures. The X-axis represents the relative mobility of each complex mannose structure through a gel. The top electroferogram is an analysis of oligomaltose for use as a mobility standard.

FIG. 10 is a schematic diagram depicting the cloning strategy for an MNN4 expression vector.

FIG. 11 is a series of electroferograms depicting N-glycan analysis of secreted glycoproteins obtained from wild-type MTLY60 cells or glycosylation mutant cells as indicated. Analysis was performed using DSA-FACE. "M5," "M6," "M7," "M8," "M9," refers to the number of mannose residues conjugated to the chitobiose core structure. "P" refers to mannoproteins containing one phosphate residue and "PP" refers to mannoproteins containing two phosphate residues. The Y-axis represents the relative fluorescence units as an indication of the amount of each of the mannose structures. The X-axis represents the relative mobility of each complex

## 13

mannose structure through a gel. The top electroferogram is an analysis of oligomaltose for use as a mobility standard.

FIGS. 12A-12H are schematic diagrams depicting the cloning strategy for an  $\alpha$ -galactosidase expression vector. The nucleotide sequences set forth in SEQ ID NOs: 76-81 are the oligonucleotides used in the cloning strategy in FIGS. 12A, 12C, and 12E.

FIG. 13 is a series of electroferograms depicting N-glycan analysis of mannoproteins and phosphomannoproteins obtained from wild-type MTLY60 cells or various clones of glycosylation mutant cells as indicated. "alg3" indicates that the cell is an ALG3 knockout. "ALG6 overexpression" indicates that the protein product of ALG6 is overexpressed in the cell. Analysis was performed using DSA-FACE. "M5," "M6," "M7," "M8," and "M9," refer to the number of mannose residues conjugated to the base N-acetylglucosamine structure. The Y-axis represents the relative fluorescence units as an indication of the amount of each of the mannose structures. The X-axis represents the relative mobility of each complex mannose structure through a polyacrylamide gel. The top electroferogram is an analysis of oligomaltose for use as a mobility standard.

FIG. 14 is a series of electroferograms depicting N-glycan analysis of mannoproteins and phosphomannoproteins obtained from wild-type MTLY60 cells or various clones of glycosylation mutant cells as indicated. "alg3" indicates that the cell is an ALG3 knockout. "ALG6 overexpression" indicates that the protein product of ALG6 is overexpressed in the cell. One peak runs at the same position as Man<sub>5</sub>GlcNAc<sub>2</sub> of the RNaseB marker and shifts with two glucose-units after  $\alpha$ -1,2-mannosidase treatment and with 4 glucose-units after alpha-mannosidase (JB) digest. This fits with a Man<sub>5</sub>GlcNAc<sub>2</sub> structure as expected. The additional two peaks run at a distance of about one and two glyco-units and are not affected by  $\alpha$ -1,2-mannosidase digestion. Both peaks shift one glucose-unit upon  $\alpha$ -mannosidase (JB) digestion. Minor shifts are due to the higher salt concentrations of the added enzymes, e.g. JB mannosidase. Analysis was performed using DSA-FACE. "M5," "M6," "M7," "M8," and "M9," refer to the number of mannose residues conjugated to the chitobiose core structure. The Y-axis represents the relative fluorescence units as an indication of the amount of each of the mannose structures. The X-axis represents the relative mobility of each complex mannose structure through a gel. The top electroferogram is an analysis of oligomaltose for use as a mobility standard.

FIG. 15 is a sequence alignment of an isolated DNA fragment (SEQ ID NO:1) sequence obtained from the unfolded protein response (UPR)-induced strain *Yarrowia lipolytica* with a genomic HAC1 DNA sequence (SEQ ID NO:5). The boxed sequence corresponds to the non-conventionally spliced intron.

FIG. 16 is a series of sequence alignments of the predicted 5' (top) and 3' (bottom) splice sites of *Pichia pastoris* (SEQ ID NO:70 and SEQ ID NO:72) and *Saccharomyces cerevisiae* (SEQ ID NO: 71 and SEQ ID NO:73). Nucleotides in bold underlined are present in the loop structure.

FIGS. 17A and 17B are two partial views of a sequence alignment of the HAC1 cDNA obtained from DTT-induced (I) (SEQ ID NO:2) and non-induced (NI) (SEQ ID NO:6) *Pichia pastoris* cultures.

FIG. 18 is a sequence alignment of the 18 amino acid C-terminal regions of *Pichia pastoris* (SEQ ID NO:74) and *Saccharomyces cerevisiae* (SEQ ID NO:75). Conserved amino acids are in bold and underlined.

FIG. 19 is a bar graph depicting the comparison of the relative expression levels of KAR2 mRNA. Clones 3, 4, and

## 14

5 (*Pichia pastoris* GSM5 cells) were grown on methanol as carbon source. "3+," "4+," and "5+" refer to the respective clones grown on methanol as carbon source, whereas "3-," "4-," and "5-" refer to the respective clones grown on glucose as carbon source. The Y-axis represents the relative expression of the KAR2 gene using real-time PCR.

FIG. 20 is a bar graph depicting the relative expression level of Kar2 and HAC1 mRNA in two *Pichia pastoris* clones (clone 6 and 8). "6+" and "8+" refer to the respective clones grown on methanol as carbon source, whereas "6-" and "8-" refer to the respective clones grown on glucose as carbon source. The Y-axis represents the relative expression of the KAR2 gene using real-time PCR.

FIG. 21 is a schematic diagram depicting the cloning strategy for a YIMNN6 expression vector.

FIG. 22 is a series of electroferograms depicting N-glycan analysis of glycoproteins obtained from *Doch1 Y. lipolytica* cells, alone, or various clones (Z3, Z4, Z5, U5, U6, and U8) of *Doch1 Y. lipolytica* expressing YIMNN6 as indicated. Analysis was performed using DSA-FACE. The Y-axis represents the relative fluorescence units as an indication of the amount of each of the mannose structures. The X-axis represents the relative mobility of each complex mannose structure through a gel. The top electroferogram is an analysis of oligomaltose for use as a mobility standard.

FIG. 23 is a schematic diagram depicting the cloning strategy for an MFManHDEL expression vector.

FIG. 24 is a series of electroferograms depicting N-glycan analysis of glycoproteins obtained from *Doch1 Y. lipolytica* cells, alone, or various clones (9, 11, 10, 3, 5, and 6) of *Doch1 Y. lipolytica* expressing MFManHDEL as indicated. Analysis was performed using DSA-FACE. The Y-axis represents the relative fluorescence units as an indication of the amount of each of the mannose structures. The X-axis represents the relative mobility of each complex mannose structure through a gel. The top electroferogram is an analysis of oligomaltose for use as a mobility standard.

FIGS. 25A and 25B are schematic diagrams depicting the cloning strategy for an LIP2preManHDEL expression vector.

FIG. 26 is a series of electroferograms depicting N-glycan analysis of glycoproteins obtained from *Doch1 Y. lipolytica* cells, alone, or various clones (1, 5, 10, and 11) of *Doch1 Y. lipolytica* expressing LIP2ManHDEL as indicated. Analysis was performed using DSA-FACE. "M5," "M6," "M7," "M8," and "M9," refer to the number of mannose residues conjugated to the chitobiose core structure. The Y-axis represents the relative fluorescence units as an indication of the amount of each of the mannose structures. The X-axis represents the relative mobility of each complex mannose structure through a gel. The top electroferogram is an analysis of oligomaltose for use as a mobility standard.

FIGS. 27A and 27B are amino acid sequences of HAC1 proteins of *Yarrowia lipolytica* (FIG. 27A; SEQ ID NO:3) and *Pichia pastoris* (FIG. 27B; SEQ ID NO:4).

FIG. 28 is a photograph of a Coomassie blue stained polyacrylamide gel depicting the results of Lip2p overexpression in various *Yarrowia lipolytica* cell (MTLY60, MTLY60 $\Delta$ alg3 and MTLY60 $\Delta$ alg3ALG6) cultures. The following samples were resolved in the gel: Lane 1 ("ladder"), a combination of proteins of known molecular weight; Lane 2 ("WT"), Lip2p protein obtained from WT *Yarrowia lipolytica* cells (MTLY60) overexpressing Lip2p; Lane 3 ("WT+PGase F"), Lip2p protein obtained from WT *Yarrowia lipolytica* cells overexpressing Lip2p and treated with PNGase F enzyme; Lane 4 ("alg3-ALG6"), Lip2p protein obtained from *Yarrowia* cells deficient in alg3 and overexpressing both Lip2p and ALG6 (MTLY60 $\Delta$ alg3ALG6); Lane 5 ("alg3-ALG6+

15

PNGase F"), Lip2p protein obtained from *Yarrowia* cells deficient in alg3 and overexpressing both Lip2p and ALG6 (MTLY60Δalg3ALG6) and treated with PNGase F enzyme; Lane 6 ("alg3"), Lip2p protein obtained from *Yarrowia lipolytica* cells deficient in alg3 and overexpressing Lip2p (MTLY60Δalg3); Lane 7 ("alg3+PNGase F"), Lip2p protein obtained from *Yarrowia lipolytica* cells deficient in alg3 and overexpressing Lip2p (MTLY60Δalg3) treated with PNGase F enzyme; Lane 8 ("WT without Lip2p overexpression"), protein obtained from MTLY60 cells; and Lane 9 ("WT without Lip2p overexpression+PNGase F"), protein obtained from MTLY60 cells and treated with PNGase F enzyme.

FIG. 29 is a series of electroferograms depicting N-glycan analysis of glycoproteins obtained from various *Yarrowia lipolytica* cells (WT (MTLY60); Δalg3; Δalg3 ALG6 overexpressing; and clones of Δalg3 overexpressing ALG6 along with the alpha subunit of glucosidase II from *Y. lipolytica* (YI) or *Typanosoma brucei* (Tb)) as indicated. Analysis was performed using DSA-FACE. "M5," "M6," "M7," "M8," and "M9," refer to the number of mannose residues conjugated to the chitobiose core structure. The Y-axis represents the relative fluorescence units as an indication of the amount of each of the mannose structures. The X-axis represents the relative mobility of each complex mannose structure through a gel. The top electroferogram is an analysis of oligomaltose for use as a mobility standard. The bottom electroferogram is an analysis of RNase B.

FIG. 30 is a series of electroferograms depicting N-glycan analysis of glycoproteins obtained from various *Yarrowia lipolytica* cells (Δalg3; Δalg3 ALG6 overexpressing; and clones of Δalg3 overexpressing ALG6 along with the alpha subunit of glucosidase II from *Y. lipolytica* (YI) containing an HDEL sequence as indicated. Analysis was performed using DSA-FACE. The Y-axis represents the relative fluorescence units as an indication of the amount of each of the mannose structures. The X-axis represents the relative mobility of each complex mannose structure through a gel.

FIG. 31 is a series of electroferograms depicting N-glycan analysis of glycoproteins obtained from various *Yarrowia lipolytica* cells (Δalg3; Δalg3 ALG6 overexpressing; and clones of Δalg3 overexpressing ALG6 along with the alpha subunit of glucosidase II from *Trypanosoma brucei* (Tb) containing an HDEL sequence) as indicated. Analysis was performed using DSA-FACE. The Y-axis represents the relative fluorescence units as an indication of the amount of each of the mannose structures. The X-axis represents the relative mobility of each complex mannose structure through a gel.

FIG. 32 is a series of electroferograms depicting N-glycan analysis of glycoproteins obtained from alg3ALG6 *Yarrowia lipolytica* cells treated in vitro with different concentrations of mutanase as indicated. Analysis was performed using DSA-FACE. The Y-axis represents the relative fluorescence units as an indication of the amount of each of the mannose structures. The X-axis represents the relative mobility of each complex mannose structure through a gel. The top electroferogram is an analysis of oligomaltose for use as a mobility standard. The bottom electroferogram is an analysis of RNase B.

FIG. 33 is a series of electroferograms depicting N-glycan analysis of glycoproteins obtained from various *Yarrowia lipolytica* cells (Δalg3; Δalg3 ALG6 overexpressing; and clones of Δalg3 overexpressing ALG6 along with the alpha subunit of glucosidase II from *Y. lipolytica* (YI.) and the beta subunit of glucosidase II from Y.I. expressed under the control of Hp4d or TEF promoters) as indicated. The Y-axis represents the relative fluorescence units as an indication of the amount of each of the mannose structures. The X-axis repre-

16

sents the relative mobility of each complex mannose structure through a gel. The top electroferogram is an analysis of oligomaltose for use as a mobility standard. The bottom electroferogram is an analysis of RNase B.

FIG. 34 is a series of electroferograms depicting N-glycan analysis of glycoproteins obtained from various *Yarrowia lipolytica* cells (Δalg3 ALG6 overexpressing; and clones of Δalg3 overexpressing ALG6 along with the HDEL-containing alpha subunit of glucosidase II from *Y. lipolytica* (YI.) and the beta subunit of glucosidase II from Y.I. expressed under the control of Hp4d or TEF promoters) as indicated. Analysis was performed using DSA-FACE. The Y-axis represents the relative fluorescence units as an indication of the amount of each of the mannose structures. The X-axis represents the relative mobility of each complex mannose structure through a gel. The top electroferogram is an analysis of oligomaltose for use as a mobility standard. The bottom electroferogram is an analysis of RNase B.

FIG. 35 is a series of electroferograms depicting N-glycan analysis of glycoproteins obtained from various *Yarrowia lipolytica* cells (Δalg3 and clones of Δalg3 overexpressing the alpha subunit of glucosidase II from *Y. lipolytica* (YI.) and the beta subunit of glucosidase II from Y.I. expressed under the control of a TEF promoter) as indicated. The Y-axis represents the relative fluorescence units as an indication of the amount of each of the mannose structures. The X-axis represents the relative mobility of each complex mannose structure through a gel. The top electroferogram is an analysis of oligomaltose for use as a mobility standard. The bottom electroferogram is an analysis of RNase B.

FIGS. 36A and 36B is the depiction of a nucleotide sequence of a cDNA encoding a mature form of *Aspergillus niger* (lacking signal peptide) glucosidase II α, which is codon-optimized cDNA for expression in *Yarrowia lipolytica*. (SEQ ID NO:7).

FIG. 37 is the depiction of a nucleotide sequence of a cDNA encoding a mature form of *Aspergillus niger* (lacking signal peptide) glucosidase II β, which is codon-optimized cDNA for expression in *Yarrowia lipolytica*. (SEQ ID NO:8).

FIG. 38 is a series of electroferograms depicting N-glycan analysis of glycoproteins obtained from various *Yarrowia lipolytica* cells (Δalg3 and ALG6 overexpressing along with the alpha subunit of glucosidase II from *Aspergillus niger* (An) expressed under the control of a TEF or hp4d promoter) as indicated. The Y-axis represents the relative fluorescence units as an indication of the amount of each of the mannose structures. The X-axis represents the relative mobility of each complex mannose structure through a gel. The top electroferogram is an analysis of oligomaltose for use as a mobility standard. The bottom electroferogram is an analysis of RNase B.

FIGS. 39A and 39B are a pair of bar graphs depicting the relative expression level (Y-axis) of the HAC1 (39A) or KAR (39B) gene in WT (MTLY60) *Yarrowia lipolytica* cells or in two clones (clone 7 and clone 2) of *Yarrowia lipolytica* cells containing a spliced form of HAC1 cDNA under the expression control of the hp4d promoter.

FIG. 40 is line graph depicting the growth of wild type *Pichia pastoris* GS115 cells transformed with an empty vector as compared to the growth of *Pichia pastoris* GS115 cells expressing the Hac1p protein.

FIG. 41 is a photograph of a Coomassie blue stained polyacrylamide gel comparing the expression level of the murine IL-10 (mIL-10) protein from a culture of *Pichia pastoris* GS115 cell cells expressing mIL-10 protein with the expression of the mIL-10 protein obtained from a culture of GS115 cells expressing mIL-10 and the spliced HAC1 protein from

17

*Pichia pastoris* under the control of an inducible promoter, AOX1. The following samples were resolved in the gel: Lane 1 ("ladder"), a combination of proteins of known molecular weight; Lane 2 ("Reference"), protein obtained from the reference mL-10 expressing *Pichia pastoris* strain (GS115); Lane 3 ("Reference"), protein obtained from the reference mL-10 expressing *Pichia pastoris* strain after PNGase F enzyme treatment of the proteins; Lane 4 ("Clone 1"), protein obtained from a mL-10 expressing *Pichia pastoris* cells inducibly expressing HAC1 protein; Lane 5 ("Clone 1"), protein obtained from a mL-10 expressing *Pichia pastoris* cells inducibly expressing HAC1 protein after treatment of the protein with PNGase F enzyme; Lane 6 ("Clone 2"), protein obtained from a mL-10 expressing *Pichia pastoris* cells inducibly expressing HAC1 protein 1; Lane 7 ("Clone 2"), protein obtained from a mL-10 expressing *Pichia pastoris* cells inducibly expressing HAC1 protein after treatment of the proteins with PNGase F enzyme.

FIG. 42 is the depiction of a nucleotide sequence of an exemplary cDNA sequence encoding a *Trichoderma reesei*  $\alpha$ -1,2 mannosidase, codon optimized for expression in *Yarrowia lipolytica* (SEQ ID NO:9) containing the LIP2 pre signal sequence.

FIG. 43 is the depiction of a nucleotide sequence of an exemplary nucleotide sequence for the GAP promoter of *Yarrowia lipolytica*. (SEQ ID NO:10).

FIGS. 44A-44C are the depiction of a nucleotide sequence of an exemplary nucleic acid sequence (SEQ ID NO:11) for the expression vector pYLHUXdL2preManHDEL, which contains a cDNA sequence encoding a *Trichoderma reesei*  $\alpha$ -1,2 mannosidase, codon optimized for expression in *Yarrowia lipolytica* and containing the LIP2 pre signal sequence.

FIGS. 45A-45C are the depiction of a nucleotide sequence of an exemplary nucleic acid sequence (SEQ ID NO:12) for the expression vector pYLHUXdL2preManHDEL, which contains a cDNA sequence encoding a *Trichoderma reesei*  $\alpha$ -1,2 mannosidase, codon optimized for expression in *Yarrowia lipolytica* and containing the LIP2 pre signal sequence.

FIGS. 46A-46C are the depiction of a nucleotide sequence of an exemplary nucleic acid sequence (SEQ ID NO:13) for the expression vector pYLPUXdL2preManHDEL, which contains a cDNA sequence encoding a *Trichoderma reesei*  $\alpha$ -1,2 mannosidase, codon optimized for expression in *Yarrowia lipolytica* and containing the LIP2 pre signal sequence.

FIGS. 47A-47C are the depiction of a nucleotide sequence of an exemplary nucleic acid sequence (SEQ ID NO:14) for the expression vector pYLTUXdL2preManHDEL, which contains a cDNA sequence encoding a *Trichoderma reesei*  $\alpha$ -1,2 mannosidase, codon optimized for expression in *Yarrowia lipolytica* and containing the LIP2 pre signal sequence.

FIG. 48 is a series of electroferograms depicting N-glycan analysis of glycoproteins obtained from *Yarrowia lipolytica* cells transformed with different expression vectors as indicated: "hp4dL2ManHDEL" (pYLHUXdL2preManHDEL, FIGS. 44A-44C); "GAPL2ManHDEL" (pYLGUXdL2preManHDEL, FIGS. 45A-45C); "TEF1L2ManHDEL" (pYLTUXdL2preManHDEL, FIGS. 47A-47C). The Y-axis represents the relative fluorescence units as an indication of the amount of each of the mannose structures. The X-axis represents the relative mobility of each complex mannose structure through a gel. The top electroferogram is an analysis of dextran for use as a mobility standard. The second electroferogram in the series is an analysis of RNase B.

FIG. 49 is a series of electroferograms depicting N-glycan analysis of glycoproteins obtained from *Yarrowia lipolytica* MTLY60  $\Delta$ och1 cells containing a stably integrated expres-

18

sion vector pYLTUXdL2preManHDEL (FIGS. 47A-47C). Glycoprotein samples were obtained from cell cultures at 24, 48, 72, and 96 hours. The top electroferogram is an analysis of dextran for use as a mobility standard. The second electroferogram in the series is an analysis of RNase B.

FIG. 50 is an exemplary nucleic acid sequence for human glucocerebrosidase (GLCM, Swiss Prot entry nr: P04062; SEQ ID NO:15), which was chemically synthesized as a codon-optimized cDNA for expression in *Yarrowia lipolytica*.

FIG. 51 is a photograph of an immunoblot depicting the mobility pattern of human glucocerebrosidase expressed in *Yarrowia lipolytica* strains MTLY60 (WT; lanes 4 and 6) and MTLY60 $\Delta$ och1 ( $\Delta$ och1; first three lanes). The molecular weight (kDa) of the proteins is depicted, by way of molecular weight markers, at the far right of the immunoblot.

FIG. 52 is an exemplary nucleic acid sequence for human erythropoietin (Epo, Swiss Prot entry nr: P01588; SEQ ID NO:16), which was chemically synthesized as a codon-optimized cDNA for expression in *Yarrowia lipolytica*.

FIG. 53 is an exemplary nucleic acid sequence for human  $\alpha$ -galactosidase A (AGAL, Swiss Prot entry nr: P06280; SEQ ID NO:17), which was chemically synthesized as a codon-optimized cDNA for expression in *Yarrowia lipolytica*.

FIG. 54 is a series of electron micrographs of wild type *Pichia pastoris* cells or *Pichia pastoris* cells overexpressing the spliced form of Hac1p protein. Discrete regions of stacked lipid membranes in the cells are boxed.

FIG. 55 is a series of electroferograms depicting N-glycan analysis of glycoproteins obtained from WT *Yarrowia lipolytica* cells (poll d) and *Yarrowia lipolytica* cells expressing a fusion protein of alpha-1,2-mannosidase and a HDEL sequence as indicated. Analysis was performed using DSA-FACE. "M5," "M6," "M7," "M8," and "M9," refer to the number of mannose residues conjugated to the chitobiose core structure. The Y-axis represents the relative fluorescence units as an indication of the amount of each of the mannose structures. The X-axis represents the relative mobility of each complex mannose structure through a gel. The top electroferogram is an analysis of RNase B. The bottom electroferogram is an analysis of oligomaltose for use as a mobility standard.

#### DETAILED DESCRIPTION

The methods and genetically engineered cells described herein can be used to produce target molecules (e.g., target protein or target dolichol) having an altered N-glycosylation form as compared to the N-glycosylation form of the target molecules produced in non-genetically engineered cells. Administration of glycosylated target molecules (e.g., glycosylated proteins) to patients having metabolic disorders (e.g., lysosomal storage disorders) has been shown to ameliorate the symptoms of the disorders. Thus, the methods and cells described are useful for the preparation of altered N-glycosylated target molecules for, inter alia, the treatment of metabolic disorders such as lysosomal storage disorders. Such altered N-glycosylation molecules are also useful in a wide-variety of other fields, e.g., the food and beverage industries; the pharmaceutical industry (e.g., as vaccines); the agriculture industry; and the chemical industry, to name a few.

#### Altered N-Glycosylation Molecule

Target molecules, as used herein, refer to any molecules that undergo altered N-glycosylation by one or more N-glycosylation activities from a genetically engineered cell (e.g., a fungal cell such as *Yarrowia lipolytica* or *Arxula adeninivorans* (or other related species dimorphic yeast) cell; a plant

cell, or an animal cell). In some embodiments, the target molecules are capable of being trafficked through one or more steps of the *Yarrowia lipolytica* or *Arxula adeninivorans* (or other related species dimorphic yeast) secretory pathway, resulting in their altered N-glycosylation by the host cell machinery. The target molecules can be endogenous or exogenous.

Target proteins, their biologically active fragments, or biologically active variants thereof, can include proteins containing additions, deletions, or substitutions as described above. Suitable target proteins include pathogen proteins (e.g., tetanus toxoid; diphtheria toxoid; viral surface proteins (e.g., cytomegalovirus (CMV) glycoproteins B, H and gCIII; human immunodeficiency virus 1 (HIV-1) envelope glycoproteins; Rous sarcoma virus (RSV) envelope glycoproteins; herpes simplex virus (HSV) envelope glycoproteins; Epstein Barr virus (EBV) envelope glycoproteins; varicella-zoster virus (VZV) envelope glycoproteins; human papilloma virus (HPV) envelope glycoproteins; Influenza virus glycoproteins; and Hepatitis family surface antigens), lysosomal proteins (e.g., glucocerebrosidase, cerebrosidase, or galactocerebrosidase), insulin, glucagon, growth factors, cytokines, chemokines, antibodies or fragments thereof, or fusions of any of the proteins to antibodies or fragments of antibodies (e.g., protein-Fc). Growth factors include, e.g., vascular endothelial growth factor (VEGF), Insulin-like growth factor (IGF), bone morphogenic protein (BMP), Granulocyte-colony stimulating factor (G-CSF), Granulocyte-macrophage colony stimulating factor (GM-CSF), Nerve growth factor (NGF); a Neurotrophin, Platelet-derived growth factor (PDGF), Erythropoietin (EPO), Thrombopoietin (TPO), Myostatin (GDF-8), Growth Differentiation factor-9 (GDF9), basic fibroblast growth factor (bFGF or FGF2), Epidermal growth factor (EGF), Hepatocyte growth factor (HGF). Cytokines include, e.g., interleukins (e.g., IL-1 to IL-33 (e.g., IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, or IL-15)). Chemokines include, e.g., I-309, TCA-3, MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES, C10, MRP-2, MARC, MCP-3, MCP-2, MRP-2, CCF18, MIP-1 $\gamma$ , Eotaxin, MCP-5, MCP-4, NCC-1, Ck $\beta$ 10, HCC-1, Leukotactin-1, LEC, NCC-4, TARC, PARC, or Eotaxin-2. Also included are tumor glycoproteins (e.g., tumor-associated antigens), for example, carcinoembryonic antigen (CEA), human mucins, HER-2/neu, and prostate-specific antigen (PSA) [R. A. Henderson and O. J. Finn, *Advances in Immunology*, 62, pp. 217-56 (1996)]. In some embodiments, the target protein can be one associated with a lysosomal storage disorder, which target proteins include, e.g., alpha-L-iduronidase, beta-D-galactosidase, beta-glucosidase, beta-hexosaminidase, beta-D-mannosidase, alpha-L-fucosidase, arylsulfatase B, arylsulfatase A, alpha-N-acetylgalactosaminidase, aspartylglucosaminidase, iduronate-2-sulfatase, alpha-glucosaminide-N-acetyltransferase, beta-D-glucuronidase, hyaluronidase, alpha-L-mannosidase, alpha-neuraminidase, phosphotransferase, acid lipase, acid ceramidase, sphingomyelinase, thioesterase, cathepsin K, and lipoprotein lipase.

Target proteins can also be fusion proteins. Fusions proteins include, e.g., a fusion of (i) any protein described herein or fragment thereof with (ii) an antibody or fragment thereof. As used herein, the term "antibody fragment" refers to an antigen-binding fragment, e.g., Fab, F(ab')<sub>2</sub>, Fv, and single chain Fv (scFv) fragments. An scFv fragment is a single polypeptide chain that includes both the heavy and light chain variable regions of the antibody from which the scFv is derived. In addition, diabodies [Poljak (1994) *Structure* 2(12):1121-1123; Hudson et al. (1999) *J. Immunol. Methods*

23(1-2):177-189, the disclosures of both of which are incorporated herein by reference in their entirety] and intrabodies [Huston et al. (2001) *Hum. Antibodies* 10(3-4):127-142; Wheeler et al. (2003) *Mol. Ther.* 8(3):355-366; Stocks (2004) *Drug Discov. Today* 9(22): 960-966, the disclosures of all of which are incorporated herein by reference in their entirety] can be used in the methods of the invention.

Target proteins can also be joined to one or more of a polymer, a carrier, an adjuvant, an immunotoxin, or a detectable (e.g., fluorescent, luminescent, or radioactive) moiety. For example, a target protein can be joined to polyethyleneglycol, which polymer moiety can be used, e.g., to increase the molecular weight of small proteins and/or increase circulation residence time.

In some embodiments, the target molecule can be, or contain, dolichol.

#### Genetically Engineered Cells

Described herein are genetically engineered cells having at least one modified N-glycosylation activity, which cells are useful for the production of one or more target molecules having an altered N-glycosylation form. Cells suitable for genetic engineering include, e.g., fungal cells (e.g., *Yarrowia lipolytica* or any other related dimorphic yeast cells described herein), plant cells, or animal cells (e.g., (nematode, insect, plant, bird, reptile, or mammal (e.g., a mouse, rat, rabbit, hamster, gerbil, dog, cat, goat, pig, cow, horse, whale, monkey, or human))). The cells can be primary cells, immortalized cells, or transformed cells. The cells can be those in an animal, e.g., a non-human mammal. Such cells, prior to the genetic engineering as specified herein, can be obtained from a variety of commercial sources and research resource facilities, such as, for example, the American Type Culture Collection (Rockville, Md.). Target molecules include proteins such as any of the target proteins described herein (see above). Target molecules also include dolichol.

Genetic engineering of a cell includes genetic modifications such as: (i) deletion of an endogenous gene encoding a protein having N-glycosylation activity; (ii) introduction of a recombinant nucleic acid encoding a mutant form of a protein (e.g., endogenous or exogenous protein) having N-glycosylation activity (i.e., expressing a mutant protein having an N-glycosylation activity); (iii) introduction or expression of an RNA molecule that interferes with the functional expression of a protein having the N-glycosylation activity; (iv) introduction of a recombinant nucleic acid encoding a wild-type (e.g., endogenous or exogenous) protein having N-glycosylation activity (i.e., expressing a protein having an N-glycosylation activity); or (v) altering the promoter or enhancer elements of one or more endogenous genes encoding proteins having N-glycosylation activity to thus alter the expression of their encoded proteins. RNA molecules include, e.g., small-interfering RNA (siRNA), short hairpin RNA (shRNA), antisense RNA, or micro RNA (miRNA). It is understood that item (ii) includes, e.g., replacement of an endogenous gene (e.g., by homologous recombination) with a gene encoding a protein having greater N-glycosylation activity relative to the endogenous gene so replaced. Genetic engineering also includes altering an endogenous gene encoding a protein having an N-glycosylation activity to produce a protein having additions (e.g., a heterologous sequence), deletions, or substitutions (e.g., mutations such as point mutations; conservative or non-conservative mutations). Mutations can be introduced specifically (e.g., site-directed mutagenesis or homologous recombination; see accompanying Examples) or can be introduced randomly (for example, cells can be chemically mutagenized as described in, e.g., Newman and Ferro-

Novick (1987) J. Cell Biol. 105(4):1587, the disclosure of which is incorporated herein by reference in its entirety.

The genetic modifications described herein can result in one or more of (i) an increase in one or more N-glycosylation activities in the genetically modified cell, (ii) a decrease in one or more N-glycosylation activities in the genetically modified cell, (iii) a change in the localization or intracellular distribution of one or more N-glycosylation activities in the genetically modified cell, or (iv) a change in the ratio of one or more N-glycosylation activities in the genetically modified cell. It is understood that an increase in the amount of an N-glycosylation activity can be due to overexpression of one or more proteins having N-glycosylation activity, an increase in copy number of an endogenous gene (e.g., gene duplication), or an alteration in the promoter or enhancer of an endogenous gene that stimulates an increase in expression of the protein encoded by the gene. A decrease in one or more N-glycosylation activities can be due to overexpression of a mutant form (e.g., a dominant negative form) of one or more proteins having N-glycosylation altering activities, introduction or expression of one or more interfering RNA molecules that reduce the expression of one or more proteins having an N-glycosylation activity, or deletion of one or more endogenous genes that encode a protein having N-glycosylation activity.

Methods of deleting or disrupting one or more endogenous genes are described in the accompanying Examples. For example, to disrupt a gene by homologous recombination, a "gene replacement" vector can be constructed in such a way to include a selectable marker gene. The selectable marker gene can be operably linked, at both 5' and 3' end, to portions of the gene of sufficient length to mediate homologous recombination. The selectable marker can be one of any number of genes which either complement host cell auxotrophy or provide antibiotic resistance, including URA3, LEU2 and HIS3 genes. Other suitable selectable markers include the CAT gene, which confers chloramphenicol resistance to yeast cells, or the lacZ gene, which results in blue colonies due to the expression of  $\beta$ -galactosidase. Linearized DNA fragments of the gene replacement vector are then introduced into the cells using methods well known in the art (see below). Integration of the linear fragments into the genome and the disruption of the gene can be determined based on the selection marker and can be verified by, for example, Southern blot analysis.

As detailed in the accompanying examples, subsequent to its use in selection, a selectable marker can be removed from the genome of the host cell by, e.g., Cre-loxP systems (see below).

Alternatively, a gene replacement vector can be constructed in such a way as to include a portion of the gene to be disrupted, which portion is devoid of any endogenous gene promoter sequence and encodes none or an inactive fragment of the coding sequence of the gene. An "inactive fragment" is a fragment of the gene that encodes a protein having, e.g., less than about 10% (e.g., less than about 9%, less than about 8%, less than about 7%, less than about 6%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, less than about 1%, or 0%) of the activity of the protein produced from the full-length coding sequence of the gene. Such a portion of the gene is inserted in a vector in such a way that no known promoter sequence is operably linked to the gene sequence, but that a stop codon and a transcription termination sequence are operably linked to the portion of the gene sequence. This vector can be subsequently linearized in the portion of the gene sequence and transformed into a cell.

By way of single homologous recombination, this linearized vector is then integrated in the endogenous counterpart of the gene.

Expression vectors can be autonomous or integrative.

A recombinant nucleic acid (e.g., one encoding a wild-type or mutant form of a protein having N-glycosylation activity) can be introduced into the cell in the form of an expression vector such as a plasmid, phage, transposon, cosmid or virus particle. The recombinant nucleic acid can be maintained extrachromosomally or it can be integrated into the yeast cell chromosomal DNA. Expression vectors can contain selection marker genes encoding proteins required for cell viability under selected conditions (e.g., URA3, which encodes an enzyme necessary for uracil biosynthesis or TRP1, which encodes an enzyme required for tryptophan biosynthesis) to permit detection and/or selection of those cells transformed with the desired nucleic acids (see, e.g., U.S. Pat. No. 4,704,362). Expression vectors can also include an autonomous replication sequence (ARS). For example, U.S. Pat. No. 4,837,148 describes autonomous replication sequences which provide a suitable means for maintaining plasmids in *Pichia pastoris*. The disclosure of U.S. Pat. No. 4,837,148 is incorporated herein by reference in its entirety.

Integrative vectors are disclosed, e.g., in U.S. Pat. No. 4,882,279 (the disclosure of which is incorporated herein by reference in its entirety). Integrative vectors generally include a serially arranged sequence of at least a first insertable DNA fragment, a selectable marker gene, and a second insertable DNA fragment. The first and second insertable DNA fragments are each about 200 (e.g., about 250, about 300, about 350, about 400, about 450, about 500, or about 1000 or more) nucleotides in length and have nucleotide sequences which are homologous to portions of the genomic DNA of the species to be transformed. A nucleotide sequence containing a gene of interest (e.g., a gene encoding a protein having N-glycosylation activity) for expression is inserted in this vector between the first and second insertable DNA fragments whether before or after the marker gene. Integrative vectors can be linearized prior to yeast transformation to facilitate the integration of the nucleotide sequence of interest into the host cell genome.

An expression vector can feature a recombinant nucleic acid under the control of a yeast (e.g., *Yarrowia lipolytica*, *Arxula adeninivorans*, or other related dimorphic yeast species) promoter, which enables them to be expressed in yeast. Suitable yeast promoters include, e.g., ADC1, TPI1, ADH2, hp4d, PDX, and Gal10 (see, e.g., Guarente et al. (1982) Proc. Natl. Acad. Sci. USA 79(23):7410) promoters. Additional suitable promoters are described in, e.g., Zhu and Zhang (1999) Bioinformatics 15(7-8):608-611 and U.S. Pat. No. 6,265,185, the disclosures of each of which are incorporated herein by reference in their entirety. Where the expression vector is to be introduced into an animal cell, such as a mammalian cell, the expression vector can feature a recombinant nucleic acid under the control of an animal cell promoter suitable for expression in the host cell of interest. Examples of mammalian promoters include, e.g., SV40 or cytomegalovirus (CMV) promoters.

A promoter can be constitutive or inducible (conditional). A constitutive promoter is understood to be a promoter whose expression is constant under the standard culturing conditions. Inducible promoters are promoters that are responsive to one or more induction cues. For example, an inducible promoter can be chemically regulated (e.g., a promoter whose transcriptional activity is regulated by the presence or absence of a chemical inducing agent such as an alcohol, tetracycline, a steroid, a metal, or other small molecule) or

physically regulated (e.g., a promoter whose transcriptional activity is regulated by the presence or absence of a physical inducer such as light or high or low temperatures). An inducible promoter can also be indirectly regulated by one or more transcription factors that are themselves directly regulated by chemical or physical cues.

Genetic engineering of a cell also includes activating an endogenous gene (e.g., a gene encoding a protein having N-glycosylation activity) that is present in the host cell, but is normally not expressed in the cells or is not expressed at significant levels in the cells. For example, a regulatory sequence (e.g., a gene promoter or an enhancer) of an endogenous gene can be modified such that the operably-linked coding sequence exhibits increased expression. Homologous recombination or targeting can be used to replace or disable the regulatory region normally associated with the gene with a regulatory sequence which causes the gene to be expressed at levels higher than evident in the corresponding non-genetically engineered cell, or causes the gene to display a pattern of regulation or induction that is different than evident in the corresponding non-genetically engineered cell. Suitable methods for introducing alterations of a regulatory sequence (e.g., a promoter or enhancer) of a gene are described in, e.g., U.S. Application Publication No. 20030147868, the disclosure of which is incorporated herein by reference in its entirety.

It is understood that other genetically engineered modifications can also be conditional. For example, a gene can be conditionally deleted using, e.g., a site-specific DNA recombinase such as the Cre-loxP system (see, e.g., Gossen et al. (2002) Ann. Rev. Genetics 36:153-173 and U.S. Application Publication No. 20060014264, the disclosures of each of which are incorporated by reference in their entirety).

A recombinant nucleic acid can be introduced into a cell described herein using a variety of methods such as the spheroplast technique or the whole-cell lithium chloride yeast transformation method. Other methods useful for transformation of plasmids or linear nucleic acid vectors into cells are described in, for example, U.S. Pat. No. 4,929,555; Hinnen et al. (1978) Proc. Nat. Acad. Sci. USA 75:1929; Ito et al. (1983) J. Bacteriol. 153:163; U.S. Pat. No. 4,879,231; and Sreekrishna et al. (1987) Gene 59:115, the disclosures of each of which are incorporated herein by reference in their entirety. Electroporation and PEG1000 whole cell transformation procedures may also be used, as described by Cregg and Russell, Methods in Molecular Biology: *Pichia* Protocols, Chapter 3, Humana Press, Totowa, N.J., pp. 27-39 (1998), the disclosure of which is incorporated herein by reference in its entirety. Transfection of animal cells can feature, for example, the introduction of a vector to the cells using calcium phosphate, electroporation, heat shock, liposomes, or transfection reagents such as FUGENE® or LIPOFECTAMINE®, or by contacting naked nucleic acid vectors with the cells in solution (see, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual Second Edition vol. 1, 2 and 3. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, N.Y., USA, November 1989; the disclosure of which is incorporated herein by reference in its entirety).

Transformed yeast cells can be selected for by using appropriate techniques including, but not limited to, culturing auxotrophic cells after transformation in the absence of the biochemical product required (due to the cell's auxotrophy), selection for and detection of a new phenotype, or culturing in the presence of an antibiotic which is toxic to the yeast in the absence of a resistance gene contained in the transformants. Transformants can also be selected and/or verified by inte-

gration of the expression cassette into the genome, which can be assessed by, e.g., Southern blot or PCR analysis.

Prior to introducing the vectors into a target cell of interest, the vectors can be grown (e.g., amplified) in bacterial cells such as *Escherichia coli* (*E. coli*). The vector DNA can be isolated from bacterial cells by any of the methods known in the art which result in the purification of vector DNA from the bacterial milieu. The purified vector DNA can be extracted extensively with phenol, chloroform, and ether, to ensure that no *E. coli* proteins are present in the plasmid DNA preparation, since these proteins can be toxic to mammalian cells.

Genetic engineering, as described herein, can be used to express (e.g., overexpress), introduce modifications into, or delete any number of genes, e.g., genes encoding proteins having N-glycosylation activity. Such genes include, e.g., ALG7, ALG13, ALG14, ALG1, ALG2, ALG11, RFT1, ALG3, ALG9, ALG12, ALG6, ALG8, ANL1, ALG10, ALG5, OST3, OST4, OST6, STT3, OST1, OST5, WBP1, SWP1, OST2, DPM1, SEC59, OCH1, MNN9, VAN1, MNN8, MNN10, MNN11, HOC1, MNN2, MNN5, MNN6, KTR1, YUR1, MNN4, KRE2, KTR2, KTR3, MNN1, MNS1, PNO1, MNN9, glucosidase I, glucosidase II, or endomannosidase. The genes encoding proteins having N-glycosylation activity can be from any species (e.g., lower eukaryotes (e.g., fungus (including yeasts) or trypanosomes), plant, or animal (e.g., insect, bird, reptile, or mammal (e.g., a rodent such as mouse or rat, dog, cat, horse, goat, cow, pig, non-human primate, or human))) containing such genes. Exemplary fungal species from which genes encoding proteins having N-glycosylation activity can be obtained include, without limitation, *Pichia anomala*, *Pichia bovis*, *Pichia canadensis*, *Pichia carsonii*, *Pichia farinose*, *Pichia fermentans*, *Pichia fluxuum*, *Pichia membranaefaciens*, *Pichia membranaefaciens*, *Candida valida*, *Candida albicans*, *Candida ascalaphidarum*, *Candida amphixiae*, *Candida Antarctica*, *Candida atlantica*, *Candida atmosphaerica*, *Candida blattae*, *Candida carpophila*, *Candida cerambycidarum*, *Candida chauliodes*, *Candida corydalis*, *Candida dosseyi*, *Candida dubliniensis*, *Candida ergatensis*, *Candida fructus*, *Candida glabrata*, *Candida fermentati*, *Candida guilliermondii*, *Candida haemulonii*, *Candida insectamens*, *Candida insectorum*, *Candida intermedia*, *Candida jeffresii*, *Candida kefir*, *Candida krusei*, *Candida lusitanae*, *Candida lyxosophila*, *Candida maltosa*, *Candida membranifaciens*, *Candida milleri*, *Candida oleophila*, *Candida oregonensis*, *Candida parapsilosis*, *Candida quercitrusa*, *Candida shehatae*, *Candida temnochilae*, *Candida tenuis*, *Candida tropicalis*, *Candida tsuchiyae*, *Candida sinolaborantium*, *Candida sojae*, *Candida viswanathii*, *Candida utilis*, *Pichia membranaefaciens*, *Pichia silvestris*, *Pichia membranaefaciens*, *Pichia chodati*, *Pichia membranaefaciens*, *Pichia membranaefaciens*, *Pichia minuscula*, *Pichia pastoris*, *Pichia pseudopoly-morpha*, *Pichia quercuum*, *Pichia robertsii*, *Pichia saitoi*, *Pichia silvestris*, *Pichia strasburgensis*, *Pichia terricola*, *Pichia vanriji*, *Pseudozyma Antarctica*, *Rhodospiridium toruloides*, *Rhodotorula glutinis*, *Saccharomyces bayanus*, *Saccharomyces bayanus*, *Saccharomyces momdshuricus*, *Saccharomyces uvarum*, *Saccharomyces bayanus*, *Saccharomyces cerevisiae*, *Saccharomyces bispurus*, *Saccharomyces chevalieri*, *Saccharomyces delbrueckii*, *Saccharomyces exiguous*, *Saccharomyces fermentati*, *Saccharomyces fragilis*, *Saccharomyces marxianus*, *Saccharomyces mellis*, *Saccharomyces rosei*, *Saccharomyces rouxii*, *Saccharomyces uvarum*, *Saccharomyces willianus*, *Saccharomycodes ludwigii*, *Saccharomycopsis capsularis*, *Saccharomycopsis fibuligera*, *Saccharomycopsis fibuligera*, *Endomyces hordei*, *Endomycopsis fobuligera*, *Saturnispora saitoi*, *Schizosac-*

*charomyces octosporus*, *Schizosaccharomyces pombe*, *Schwanniomyces occidentalis*, *Torulaspora delbrueckii*, *Torulaspora delbrueckii*, *Saccharomyces dairensis*, *Torulaspora delbrueckii*, *Torulaspora fermentati*, *Saccharomyces fermentati*, *Torulaspora delbrueckii*, *Torulaspora rosei*, *Saccharomyces rosei*, *Torulaspora delbrueckii*, *Saccharomyces rosei*, *Torulaspora delbrueckii*, *Saccharomyces delbrueckii*, *Torulaspora delbrueckii*, *Saccharomyces delbrueckii*, *Zygosaccharomyces mongolicus*, *Dorulaspora globosa*, *Debaryomyces globosus*, *Torulopsis globosa*, *Trichosporon cutaneum*, *Trigonopsis variabilis*, *Williopsis californica*, *Williopsis saturnus*, *Zygosaccharomyces bisporus*, *Zygosaccharomyces bisporus*, *Debaryomyces dispersa*, *Saccharomyces bisporus*, *Zygosaccharomyces bisporus*, *Saccharomyces bisporus*, *Zygosaccharomyces mellis*, *Zygosaccharomyces priorianus*, *Zygosaccharomyces rouxiim*, *Zygosaccharomyces rouxii*, *Zygosaccharomyces barkeri*, *Saccharomyces rouxii*, *Zygosaccharomyces rouxii*, *Zygosaccharomyces major*, *Saccharomyces rousii*, *Pichia anomala*, *Pichia bovis*, *Pichia Canadensis*, *Pichia carsonii*, *Pichia farinose*, *Pichia fermentans*, *Pichia fluxuum*, *Pichia membranaefaciens*, *Pichia pseudopolymorpha*, *Pichia quercuum*, *Pichia robertsii*, *Pseudozyma Antarctica*, *Rhodospiridium toruloides*, *Rhodospiridium toruloides*, *Rhodotorula glutinis*, *Saccharomyces bayanus*, *Saccharomyces bayanus*, *Saccharomyces bisporus*, *Saccharomyces cerevisiae*, *Saccharomyces chevalieri*, *Saccharomyces delbrueckii*, *Saccharomyces fermentati*, *Saccharomyces fragilis*, *Saccharomycodes ludwigii*, *Schizosaccharomyces pombe*, *Schwanniomyces occidentalis*, *Torulaspora delbrueckii*, *Torulaspora globosa*, *Trigonopsis variabilis*, *Williopsis californica*, *Williopsis saturnus*, *Zygosaccharomyces bisporus*, *Zygosaccharomyces mellis*, *Zygosaccharomyces rouxii*, or any other fungi (e.g., yeast) known in the art or described herein. Exemplary lower eukaryotes also include various species of *Aspergillus* including, but not limited to, *Aspergillus caesiellus*, *Aspergillus candidus*, *Aspergillus carneus*, *Aspergillus clavatus*, *Aspergillus deflectus*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus glaucus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus oryzae*, *Aspergillus parasiticus*, *Aspergillus penicilloides*, *Aspergillus restrictus*, *Aspergillus sojae*, *Aspergillus sydowii*, *Aspergillus tamari*, *Aspergillus terreus*, *Aspergillus ustus*, or *Aspergillus versicolor*. Exemplary protozoal genera from which genes encoding proteins having N-glycosylation activity can be obtained include, without limitation, *Blastocrithidia*, *Crithidia*, *Endotrypanum*, *Herpetomonas*, *Leishmania*, *Leptomonas*, *Phytomonas*, *Trypanosoma* (e.g., *T. brucei*, *T. gambiense*, *T. rhodesiense*, and *T. cruzi*), and *Wallaceina*.

It is understood that genetic engineering, as described herein, can be used to express (e.g., overexpress), introduce modifications into, or delete any number of genes (e.g., genes encoding proteins having N-glycosylation activity) and/or any combination of one or more (e.g., two, three, four, five, six, seven, eight, nine, 10, 11, 12, 15, or 20 or more) of any of the genes recited herein.

In some embodiments, the genetically engineered cell lacks the ALG3 (Genbank® Accession Nos: XM\_503488, Genolevures Ref: YALIOE03190g) gene or gene product (e.g., mRNA or protein) thereof. In some embodiments, the genetically engineered cell expresses (e.g., overexpresses) the ALG6 (Genbank® Accession Nos: XM\_502922, Genolevures Ref: YALIOD17028g) protein. In some embodiments, the genetically engineered cell expresses the MNN4 gene (Genbank® Accession Nos: XM\_503217, Genolevures Ref: YALIOD24101g). In some embodiments, the genetically engineered cell lacks the OCH1 and/or MNN9 gene or gene

products (e.g., mRNA or protein) thereof. In some embodiments, the genetically engineered cell does not lack the OCH1 gene or a gene product (e.g., mRNA or protein) thereof. In some embodiments, the genetically engineered cell expresses an alpha or beta subunit (or both the alpha and the beta subunit) of a glucosidase II such as the glucosidase II of *Yarrowia lipolytica* or *Trypanosoma brucei*. In some embodiments, the genetically engineered cell expresses a mutanase such as the mutanase of *T. harzianum*. In some embodiments, the genetically engineered cell can have any combination of these modifications.

For example, in some embodiments, the genetically engineered cell can lack the ALG3 (e.g., the ALG3 gene exemplified by Genbank® Accession Nos: XM\_503488, Genolevures Ref: YALIOE03190g) gene or gene product (e.g., mRNA or protein) thereof; can overexpress the ALG6 (e.g., the ALG6 as exemplified by Genbank® Accession Nos: XM\_502922, Genolevures Ref: YALIOD17028g) protein; can overexpress one or both of the alpha and the beta subunit of a glucosidase II (such as the glucosidase II of *Yarrowia lipolytica*, *Trypanosoma brucei*, or any other species described herein); can overexpress an alpha-1,2-mannosidase; and overexpress one or more (and any combination) of the following: a glycosidase, a glycosyltransferase, a sugar-nucleotide transporter, a sugar-nucleotide modifying enzyme. In some embodiments, the genetically engineered cell does not lack the OCH1 gene or a gene product (e.g., mRNA or protein) thereof.

In some embodiments, the genetically modified cell can contain a mannosidase activity (e.g., an  $\alpha$ -mannosidase activity). The mannosidase activity can be targeted to the endoplasmic reticulum. The mannosidase can have a pH optimum at least below 7.5 (e.g., at least below 7.4, at least below 7.3, at least below 7.2, at least below 7.1, at least below 7.0, at least below 6.9, at least below 6.8, at least 6.7, at least below 6.6, at least below 6.5, at least 6.4, at least below 6.3, at least below 6.2, at least below 6.1, at least below 6.0, at least below 5.9, at least below 5.8, at least below 5.7, at least below 5.6, at least below 5.5, at least below 5.4, at least below 5.3, at least below 5.2, at least below 5.1, at least below 5.0, at least below 4.9, at least below 4.8, or at least below 4.7).

The mannosidase can be MNS1.

For example, the genetically engineered cell can overexpress a mannosidase (e.g., an alpha-1,2-mannosidase or any other mannosidase described herein), but not lack the OCH1 gene or a gene product (e.g., mRNA or protein) thereof. The mannosidase can be a wild-type form of the protein or can be a mutant form such as a fusion protein containing a mannosidase and an HDEL ER-retention amino acid sequence (see Examples). (It is understood that any protein having N-glycosylation activity can be engineered into a fusion protein comprising an HDEL sequence).

In some embodiments, the genetically modified cell can contain an activity capable of promoting mannosyl phosphorylation of the altered N-glycosylation form of the target molecule. For example, a nucleic acid encoding an activity that promotes phosphorylation of N-glycans (e.g. MNN4, MNN6, PNO1) can be introduced in the genetically engineered cell, which cell is capable of increasing phosphorylating the N-glycosylation of the target molecule.

In some embodiments, the genetically modified cell can contain an activity capable of removing mannose residues that cap phosphorylation (e.g., a mannosidase such as the one from Jack Bean) from the altered N-glycosylation molecules.

In some embodiments, the genetically modified cell is capable of removing glucose residues from Man<sub>5</sub>GlcNAc<sub>2</sub>. For example, the genetically modified cell can overexpress a

protein having  $\alpha$ -1,3-glucosidase activity such as, but not limited to, a mutanase or one or both of the alpha and beta subunit of a glucosidase II (such as the glucosidase II of *Yarrowia lipolytica*, *Trypanosoma brucei*, or any other fungal species described herein).

In embodiments where a protein having N-glycosylation activity is derived from a cell that is of a different type (e.g., of a different species) than the cell into which the protein is to be expressed, a nucleic acid encoding the protein can be codon-optimized for expression in the particular cell of interest. For example, a nucleic acid encoding a protein having N-glycosylation from *Trypanosoma brucei* can be codon-optimized for expression in a yeast cell such as *Yarrowia lipolytica*. Such codon-optimization can be useful for increasing expression of the protein in the cell of interest. Methods for codon-optimizing a nucleic acid encoding a protein are known in the art and described in, e.g., Gao et al. (Biotechnol. Prog. (2004) 20(2): 443-448), Kotula et al. (Nat. Biotechnol. (1991) 9, 1386-1389), and Bennetzen et al. (J. Biol. Chem. (1982) 257(6):2036-3031).

A cell can also be genetically engineered to produce predominantly N-glycans that are intermediates of a mammalian (e.g., human) glycosylation pathway. For example, one or more nucleic acids encoding human proteins having N-glycosylation activity can be introduced into the cell. In some embodiments, human proteins can be introduced into the cell and one or more endogenous yeast proteins having N-glycosylation activity can be suppressed (e.g., deleted or mutated). Techniques for "humanizing" a fungal glycosylation pathway are described in, e.g., Choi et al. (2003) Proc. Natl. Acad. Sci. USA 100(9):5022-5027; Verveken et al. (2004) Appl. Environ. Microb. 70(5):2639-2646; and Gerngross (2004) Nature Biotech. 22(11):1410-1414, the disclosures of each of which are incorporated herein by reference in their entirety.

Where the genetic engineering involves, e.g., changes in the expression of a protein or expression of an exogenous protein (including a mutant form of an endogenous protein), a variety of techniques can be used to determine if the genetically engineered cells express the protein. For example, the presence of mRNA encoding the protein or the protein itself can be detected using, e.g., Northern Blot or RT-PCR analysis or Western Blot analysis, respectively. The intracellular localization of a protein having N-glycosylation activity can be analyzed by using a variety of techniques, including subcellular fractionation and immunofluorescence.

Additional genetic modifications and methods for introducing them into any of the cells described herein can be adapted from the disclosures of, e.g., U.S. Pat. Nos. 7,029, 872; 5,272,070; and 6,803,225; and U.S. Application Publication Nos. 20050265988, 20050064539, 20050170452, and 20040018588, the disclosures of each of which are incorporated herein by reference in their entirety.

While the engineering steps performed in dimorphic yeast species to achieve in vivo production of the  $\text{Man}_5\text{GlcNAc}_2$  and  $\text{Man}_3\text{GlcNAc}_2$  can be different from the engineering steps performed in other yeast species, it will be clear to those skilled in the art that the engineering techniques to produce modified glycoproteins (with the  $\text{Man}_5\text{GlcNAc}_2$  and  $\text{Man}_3\text{GlcNAc}_2$  core N-glycan structures) in dimorphic yeasts in vivo can be adapted by routine experimentation from the methods disclosed in, inter alia, U.S. Pat. No. 7,326,681 and U.S. Publication Nos. 20040018590, 20060040353, and 20060286637 (the disclosure of each of which is incorporated by reference in its entirety). The adapted methods can thus be used to achieve production of glycoproteins modified with human-type hybrid and complex N-glycans. These complex N-glycans can have 2 to 5 branches initiated with a GlcNAc

residue onto the above-named core glycans, which can be further extended, e.g., with galactose, fucose and sialic acid residues.

In some embodiments, the mutant or wild-type proteins having N-glycosylation activity can be isolated from the genetically engineered cells using standard techniques. For example, following the expression of a mutant or wild-type protein in the genetically engineered cell, the protein can be isolated from the cell itself or from the media in which the cell was cultured. Methods of isolating proteins are known in the art and include, e.g., liquid chromatography (e.g., HPLC), affinity chromatography (e.g., metal chelation or immunoaffinity chromatography), ion-exchange chromatography, hydrophobic-interaction chromatography, precipitation, or differential solubilization.

In some embodiments, the isolated proteins having N-glycosylation activity can be frozen, lyophilized, or immobilized and stored under appropriate conditions, which allow the proteins to retain activity.

The disclosure also provides a substantially pure culture of any of the genetically engineered cells described herein. As used herein, a "substantially pure culture" of a genetically engineered cell is a culture of that cell in which less than about 40% (i.e., less than about: 35%; 30%; 25%; 20%; 15%; 10%; 5%; 2%; 1%; 0.5%; 0.25%; 0.1%; 0.01%; 0.001%; 0.0001%; or even less) of the total number of viable cells in the culture are viable cells other than the genetically engineered cell, e.g., bacterial, fungal (including yeast), mycoplasmal, or protozoan cells. The term "about" in this context means that the relevant percentage can be 15% percent of the specified percentage above or below the specified percentage. Thus, for example, about 20% can be 17% to 23%. Such a culture of genetically engineered cells includes the cells and a growth, storage, or transport medium. Media can be liquid, semi-solid (e.g., gelatinous media), or frozen. The culture includes the cells growing in the liquid or in/on the semi-solid medium or being stored or transported in a storage or transport medium, including a frozen storage or transport medium. The cultures are in a culture vessel or storage vessel or substrate (e.g., a culture dish, flask, or tube or a storage vial or tube).

The genetically engineered cells described herein can be stored, for example, as frozen cell suspensions, e.g., in buffer containing a cryoprotectant such as glycerol or sucrose, as lyophilized cells. Alternatively, they can be stored, for example, as dried cell preparations obtained, e.g., by fluidized bed drying or spray drying, or any other suitable drying method.

#### Methods of Producing Altered N-Glycosylation Molecules

Described herein are methods of producing an altered N-glycosylation form of a target molecule. The methods generally involve the step of contacting a target molecule with one or more N-glycosylation activities from a genetically engineered cell (e.g., a fungal cell (e.g., *Yarrowia lipolytica*, *Arxula adeninivorans*, or any other related dimorphic yeast cells described herein), a plant cell, or an animal cell (e.g., nematode, insect, plant, bird, reptile, or mammal (e.g., a mouse, rat, rabbit, hamster, gerbil, dog, cat, goat, pig, cow, horse, whale, monkey, or human))). The methods can be cell-based or non-cell based.

Cell based methods can include the steps of introducing into a cell (e.g., a fungal cell (e.g., *Yarrowia lipolytica*, *Arxula adeninivorans*, or any other related dimorphic yeast cells described herein), a plant cell, or an animal cell) genetically engineered to have at least one modified N-glycosylation activity a nucleic acid encoding a target molecule subject to N-glycosylation in the cell, wherein the cell produces the target molecule in an altered N-glycosylation form. The target

molecule can be, e.g., a protein such as any of the target proteins described herein. In embodiments where the target protein is a lipid, the nucleic acid can be one encoding one or more enzymes which promote the synthesis of the lipid.

The types of modifications produced by the genetic engineering of the cells are described herein (see the accompanying Examples and "Genetically Engineered Cells" above).

Methods for introducing a nucleic acid are known in the art and are described in the accompanying Examples and above.

Introduction or expression of a target molecule (e.g., a target protein) into a genetically engineered cell can result in the trafficking of the target molecule through the endoplasmic reticulum and/or Golgi apparatus of the cell, thereby producing an altered N-glycosylation form of the target molecule.

Following the processing of the target molecule (e.g., in the genetically modified cell), the altered N-glycosylation form of the target molecule (e.g., the target protein) can contain one or more N-glycan structures. For example, the altered form of the target molecule can contain one or more specific N-glycan structures such as  $\text{Man}_5\text{GlcNAc}_2$  (structural formula I or VII; FIG. 4A),  $\text{Man}_8\text{GlcNAc}_2$  (structural formula I; FIG. 4A),  $\text{Man}_5\text{GlcNAc}_2$  (structural formula II; FIG. 4A),  $\text{Man}_3\text{GlcNAc}_2$  (structural formula XIV; FIG. 4B),  $\text{Glc}_1\text{Man}_5\text{GlcNAc}_2$  (structural formula VIII; FIG. 4B), or  $\text{Glc}_2\text{Man}_5\text{GlcNAc}_2$  (structural formula IX; FIG. 4B) ("Man" is mannose; "Glc" is glucose; and "GlcNAc" is N-acetylglucosamine).

The target molecules having altered N-glycosylation produced from the genetically engineered cells can be homogeneous (i.e., all altered N-glycosylation molecules containing the same specific N-glycan structure) or can be substantially homogeneous. By "substantially homogeneous" is meant that the altered target molecules are at least about 25% (e.g., at least about 27%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95%, or at least about 99%) of the target molecules having altered N-glycosylation produced by the genetically engineered cell.

Where the genetically engineered cell includes one or more N-glycosylation activities that effect the phosphorylation of an N-glycan, an altered N-glycosylation form of a target molecule can have at least about 25% (e.g., at least about 27%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, or at least about 80%) of its mannosyl residues phosphorylated.

Where any of the genetic modifications of the genetically engineered cell are inducible or conditional on the presence of an inducing cue (e.g., a chemical or physical cue), the genetically engineered cell can, optionally, be cultured in the presence of an inducing agent before, during, or subsequent to the introduction of the nucleic acid. For example, following introduction of the nucleic acid encoding a target protein, the cell can be exposed to a chemical inducing agent that is capable of promoting the expression of one or more proteins having N-glycosylation activity. Where multiple inducing cues induce conditional expression of one or more proteins having N-glycosylation activity, a cell can be contacted with multiple inducing agents.

Following processing by one or more N-glycosylation activities, the altered target molecule can be isolated. The altered target molecule can be maintained within the yeast cell and released upon cell lysis or the altered target molecule can be secreted into the culture medium via a mechanism

provided by a coding sequence (either native to the exogenous nucleic acid or engineered into the expression vector), which directs secretion of the molecule from the cell. The presence of the altered target molecule in the cell lysate or culture medium can be verified by a variety of standard protocols for detecting the presence of the molecule. For example, where the altered target molecule is a protein, such protocols can include, but are not limited to, immunoblotting or radioimmunoprecipitation with an antibody specific for the altered target protein (or the target protein itself), binding of a ligand specific for the altered target protein (or the target protein itself), or testing for a specific enzyme activity of the altered target protein (or the target protein itself).

In some embodiments, the isolated altered target molecules can be frozen, lyophilized, or immobilized and stored under appropriate conditions, e.g., which allow the altered target molecules to retain biological activity.

The altered N-glycosylation form of the target molecule can be further processed in vivo (e.g., in the genetically engineered cell) or can be processed in vitro following isolation from the genetically engineered cell or cell medium. The further processing can include modifications of one or more N-glycan residues of the altered target molecule or modifications to the altered target molecule other than to its N-glycan residues. The additional processing of the altered target molecule can include the addition (covalent or non-covalent joining) of a heterologous moiety such as a polymer or a carrier. The further processing can also involve enzymatic or chemical treatment of the altered target molecule. Enzymatic treatment can involve contacting the altered target molecule with one or more of a glycosidase (e.g., mannosidase or mannanase), a phosphodiesterase, a phospholipase, a glycosyltransferase, or a protease for a time sufficient to induce modification of the altered target molecule. Enzymatic treatment can also involve contacting the altered target molecule with an enzyme capable of removing one or more glucose residues from  $\text{Man}_5\text{GlcNAc}_2$  such as, but not limited to, a mannosidase or one or both of the alpha and beta subunit of a glucosidase II. Chemical treatment can, for example, involve contacting the altered target molecule with an acid such as hydrofluoric acid for a time sufficient to induce modification of the altered target molecule. Hydrofluoric acid treatment under certain conditions specifically removes the mannose residues that are phosphodiester-linked to glycans, while leaving the phosphate on the glycan. An altered target molecule can be further processed by addition or removal of a phosphate group from one or more N-glycans. For example, a altered target molecule can be contacted with a mannosyl kinase or a mannosyl phosphatase.

In some embodiments, any of the altered target molecules described herein, following isolation, can be attached to a heterologous moiety, e.g., using enzymatic or chemical means. A "heterologous moiety" refers to any constituent that is joined (e.g., covalently or non-covalently) to the altered target molecule, which constituent is different from a constituent originally present on the altered target molecule. Heterologous moieties include, e.g., polymers, carriers, adjuvants, immunotoxins, or detectable (e.g., fluorescent, luminescent, or radioactive) moieties. In some embodiments, an additional N-glycan can be added to the altered target molecule.

It is understood that a target molecule can be, but need not be, processed in a genetically engineered cell. For example, the disclosure also features cell-free methods of producing a target molecule having an altered N-glycosylation form, which methods include the step of contacting a target molecule under N-glycosylation conditions with a cell lysate

prepared from a cell (e.g., a fungal cell (e.g., *Yarrowia lipolytica*, *Arxula adeninivorans*, or any other related dimorphic yeast cells described herein), a plant cell, or an animal cell (e.g., nematode, insect, plant, bird, reptile, or mammal (e.g., a mouse, rat, rabbit, hamster, gerbil, dog, cat, goat, pig, cow, horse, whale, monkey, or human)) genetically engineered to have at least one modified N-glycosylation activity, wherein the contacting of the target molecule to the cell lysate produces an altered N-glycosylation form of the target molecule.

By "N-glycosylation conditions" is meant that a mixture (e.g., of target molecule and cell lysate) is incubated under conditions that allow for altered N-glycosylation (as described above).

Suitable methods for obtaining cell lysates that preserve the activity or integrity of one or more N-glycosylation activities in the lysate can include the use of appropriate buffers and/or inhibitors, including nuclease, protease and phosphatase inhibitors that preserve or minimize changes in N-glycosylation activities in the cell lysate. Such inhibitors include, for example, chelators such as ethylenediamine tetraacetic acid (EDTA), ethylene glycol bis(P-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, antipain and the like, and phosphatase inhibitors such as phosphate, sodium fluoride, vanadate and the like. Inhibitors can be chosen such that they do not interfere with or only minimally adversely affect the N-glycosylation activity, or activities, of interest. Appropriate buffers and conditions for obtaining lysates containing enzymatic activities are described in, e.g., Ausubel et al. Current Protocols in Molecular Biology (Supplement 47), John Wiley & Sons, New York (1999); Harlow and Lane, Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory Press (1988); Harlow and Lane, Using Antibodies: A Laboratory Manual, Cold Spring Harbor Press (1999); Tietz Textbook of Clinical Chemistry, 3rd ed. Burtis and Ashwood, eds. W.B. Saunders, Philadelphia, (1999).

A cell lysate can be further processed to eliminate or minimize the presence of interfering substances, as appropriate. If desired, a cell lysate can be fractionated by a variety of methods well known to those skilled in the art, including subcellular fractionation, and chromatographic techniques such as ion exchange, hydrophobic and reverse phase, size exclusion, affinity, hydrophobic charge-induction chromatography, and the like (see, e.g., Scopes, Protein Purification: Principles and Practice, third edition, Springer-Verlag, New York (1993); Burton and Harding, J. Chromatogr. A 814:71-81 (1998)).

In some embodiments, a cell lysate can be prepared in which whole cellular organelles remain intact and/or functional. For example, a lysate can contain one or more of intact rough endoplasmic reticulum, intact smooth endoplasmic reticulum, or intact Golgi apparatus. Suitable methods for preparing lysates containing intact cellular organelles and testing for the functionality of the organelles are described in, e.g., Moreau et al. (1991) J. Biol. Chem. 266(7):4329-4333; Moreau et al. (1991) J. Biol. Chem. 266(7):4322-4328; Rexach et al. (1991) J. Cell Biol. 114(2):219-229; and Paulik et al. (1999) Arch. Biochem. Biophys. 367(2):265-273; the disclosures of each of which are incorporated herein by reference in their entirety.

The disclosure also provides methods of producing a target molecule having an altered N-glycosylation form that includes the step of contacting a target molecule under N-glycosylation conditions with one or more isolated proteins having N-glycosylation activity, wherein contacting the target molecule with the one or more proteins having N-glycosylation activity produces an altered N-glycosylation form of the

target molecule and wherein the one or more proteins having N-glycosylation activity are prepared from a cell (e.g., a fungal cell (e.g., *Yarrowia lipolytica*, *Arxula adeninivorans*, or any other related dimorphic yeast cells described herein), a plant cell, or an animal cell (e.g., nematode, insect, plant, bird, reptile, or mammal (e.g., a mouse, rat, rabbit, hamster, gerbil, dog, cat, goat, pig, cow, horse, whale, monkey, or human)) genetically engineered to have at least one modified N-glycosylation activity.

One of more proteins having N-glycosylation activity can be purified using standard techniques as described above. A target molecule can be contacted with one or more proteins in a suitable buffer for a time sufficient to induce modification of the target molecule as described in, e.g., Lee and Park (2002) 30(6):716-720 and Fujita and Takegawa (2001) Biochem. Biophys. Res. Commun. 282(3):678-682, the disclosures of which are incorporated herein by reference in their entirety.

In some embodiments, the target molecule can be contacted with just one protein having N-glycosylation activity. In some embodiments, the target molecule can be contacted with more than one protein having N-glycosylation activity. The target molecule can be contacted with more than one protein at the same time or sequentially. Where the target molecule is contacted sequentially to more than one protein having N-glycosylation activity, the target molecule can, but need not, be purified after one or more steps. That is, a target molecule can be contacted with protein activity A, then purified before contacting the molecule to protein activity B, and so on.

In some embodiments of the cell free methods, it can be advantageous to link the target molecule to a solid-phase support prior to contacting the target molecule with one or more N-glycosylation activities. Such linkage can allow for easier purification following the N-glycosylation modifications. Suitable solid-phase supports include, but are not limited to, multi-well assay plates, particles (e.g., magnetic or encoded particles), a column, or a membrane.

Methods for detecting N-glycosylation (e.g., altered N-glycosylation) of a target molecule include DNA sequencer-assisted (DSA), fluorophore-assisted carbohydrate electrophoresis (FACE) (as described in the accompanying Examples) or surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) and. For example, an analysis can utilize DSA-FACE in which, for example, glycoproteins are denatured followed by immobilization on, e.g., a membrane. The glycoproteins can then be reduced with a suitable reducing agent such as dithiothreitol (DTT) or  $\beta$ -mercaptoethanol. The sulfhydryl groups of the proteins can be carboxylated using an acid such as iodoacetic acid. Next, the N-glycans can be released from the protein using an enzyme such as N-glycosidase F. N-glycans, optionally, can be reconstituted and derivatized by reductive amination. The derivatized N-glycans can then be concentrated. Instrumentation suitable for N-glycan analysis includes, e.g., the ABI PRISM® 377 DNA sequencer (Applied Biosystems). Data analysis can be performed using, e.g., GENESCAN® 3.1 software (Applied Biosystems). Optionally, isolated mannoproteins can be further treated with one or more enzymes to confirm their N-glycan status. Exemplary enzymes include, e.g.,  $\alpha$ -mannosidase or  $\alpha$ -1,2 mannosidase, as described in the accompanying Examples. Additional methods of N-glycan analysis include, e.g., mass spectrometry (e.g., MALDI-TOF-MS), high-pressure liquid chromatography (HPLC) on normal phase, reversed phase and ion exchange chromatography (e.g., with pulsed amperometric detection when glycans are not labeled and with UV absorbance or fluorescence if glycans are appropriately

labeled). See also Callewaert et al. (2001) *Glycobiology* 11(4):275-281 and Freire et al. (2006) *Bioconjug. Chem.* 17(2):559-564, the disclosures of each of which are incorporated herein by reference in their entirety.

#### Disorders Treatable by Altered N-Glycosylation Molecules

The isolated, altered N-glycosylation molecules (e.g., the altered N-glycosylation proteins or dolichol) described herein can be used to treat a variety of disorders, which disorders are treatable by administration of one or more altered N-glycosylation molecules (e.g., a protein having altered N-glycosylation). Examples of some specific medical conditions that can be treated or prevented by administration of an altered N-glycosylation molecule (e.g., an altered N-glycoprotein or an altered N-glycosylated dolichol) are reviewed in the following sections.

##### (i) Metabolic Disorders

A metabolic disorder is one that affects the production of energy within individual human (or animal) cells. Most metabolic disorders are genetic, though some can be "acquired" as a result of diet, toxins, infections, etc. Genetic metabolic disorders are also known as inborn errors of metabolism. In general, the genetic metabolic disorders are caused by genetic defects that result in missing or improperly constructed enzymes necessary for some step in the metabolic process of the cell. The largest classes of metabolic disorders are disorders of carbohydrate metabolism, disorders of amino acid metabolism, disorders of organic acid metabolism (organic acidurias), disorders of fatty acid oxidation and mitochondrial metabolism, disorders of porphyrin metabolism, disorders of purine or pyrimidine metabolism, disorders of steroid metabolism disorders of mitochondrial function, disorders of peroxisomal function, and lysosomal storage disorders (LSDs).

Examples of metabolic disorders that can be treated through the administration of one or more altered N-glycosylation molecules (or pharmaceutical compositions of the same) described herein can include, e.g., hereditary hemochromatosis, oculocutaneous albinism, protein C deficiency, type I hereditary angioedema, congenital sucrase-isomaltase deficiency, Crigler-Najjar type II, Laron syndrome, hereditary Myeloperoxidase, primary hypothyroidism, congenital long QT syndrome, tyroxine binding globulin deficiency, familial hypercholesterolemia, familial chylomicronemia, abeta-lipoproteinemia, low plasma lipoprotein A levels, hereditary emphysema with liver injury, congenital hypothyroidism, osteogenesis imperfecta, hereditary hypofibrinogenemia, alpha-1 antitrypsin deficiency, nephrogenic diabetes insipidus, neurohypophyseal diabetes insipidus, adenosine deaminase deficiency, Pelizaeus Merzbacher disease, von Willebrand disease type IIA, combined factors V and VIII deficiency, spondylo-epiphyseal dysplasia tarda, choroideremia, I cell disease, Batten disease, ataxia telangiectasia, ADPKD-autosomal dominant polycystic kidney disease, microvillus inclusion disease, tuberous sclerosis, oculocerebro-renal syndrome of Lowe, amyotrophic lateral sclerosis, myelodysplastic syndrome, Bare lymphocyte syndrome, Tangier disease, familial intrahepatic cholestasis, X-linked adrenoleukodystrophy, Scott syndrome, Hermansky-Pudlak syndrome types 1 and 2, Zellweger syndrome, rhizomelic chondrodysplasia punctata, autosomal recessive primary hyperoxaluria, Mohr Tranebjærsg syndrome, spinal and bulbar muscular atrophy, primary ciliary dyskinesia (Kartagener's syndrome), gigantism and acromegaly, galactorrhea, Addison's disease, adrenal virilism, Cushing's syndrome, ketoacidosis, primary or secondary aldosteronism, Miller Dieker syndrome, lissencephaly, motor neuron disease, Usher's syndrome, Wiskott-Aldrich syndrome, Optic

syndrome, Huntington's disease, hereditary pancreatitis, anti-phospholipid syndrome, overlap connective tissue disease, Sjögren's syndrome, stiff-man syndrome, Brugada syndrome, congenital nephritic syndrome of the Finnish type, Dubin-Johnson syndrome, X-linked hypophosphatemia, Pendred syndrome, persistent hyperinsulinemic hypoglycemia of infancy, hereditary spherocytosis, aceruloplasminemia, infantile neuronal ceroid lipofuscinosis, pseudoachondroplasia and multiple epiphyseal, Stargardt-like macular dystrophy, X-linked Charcot-Marie-Tooth disease, autosomal dominant retinitis pigmentosa, Wolcott-Rallison syndrome, Cushing's disease, limb-girdle muscular dystrophy, mucopolysaccharidosis type IV, hereditary familial amyloidosis of Finnish, Anderson disease, sarcoma, chronic myelomonocytic leukemia, cardiomyopathy, faciogenital dysplasia, Torsion disease, Huntington and spinocerebellar ataxias, hereditary hyperhomocysteinemia, polyneuropathy, lower motor neuron disease, pigmented retinitis, seronegative polyarthritis, interstitial pulmonary fibrosis, Raynaud's phenomenon, Wegner's granulomatosis, preteinuria, CDG-Ia, CDG-Ib, CDG-Ic, CDG-Id, CDG-Ie, CDG-If, CDG-IIa, CDG-IIb, CDG-IIc, CDG-IId, Ehlers-Danlos syndrome, multiple exostoses, Griscelli syndrome (type 1 or type 2), or X-linked non-specific mental retardation. In addition, metabolic disorders can also include lysosomal storage disorders such as, but not limited to, Fabry disease, Farber disease, Gaucher disease, GM<sub>1</sub>-gangliosidosis, Tay-Sachs disease, Sandhoff disease, GM<sub>2</sub> activator disease, Krabbe disease, metachromatic leukodystrophy, Niemann-Pick disease (types A, B, and C), Hurler disease, Scheie disease, Hunter disease, Sanfilippo disease, Morquio disease, Maroteaux-Lamy disease, hyaluronidase deficiency, aspartylglucosaminuria, fucosidosis, mannosidosis, Schindler disease, sialidosis type 1, Pompe disease, Pycnodysostosis, ceroid lipofuscinosis, cholesterol ester storage disease, Wolman disease, Multiple sulfatase deficiency, galactosialidosis, mucopolidosis (types II, III, and IV), cystinosis, sialic acid storage disorder, chylomicron retention disease with Marinesco-Sjögren syndrome, Hermansky-Pudlak syndrome, Chediak-Higashi syndrome, Danon disease, or Geleophysic dysplasia.

Symptoms of a metabolic disorder are numerous and diverse and can include one or more of, e.g., anemia, fatigue, bruising easily, low blood platelets, liver enlargement, spleen enlargement, skeletal weakening, lung impairment, infections (e.g., chest infections or pneumonias), kidney impairment, progressive brain damage, seizures, extra thick meconium, coughing, wheezing, excess saliva or mucous production, shortness of breath, abdominal pain, occluded bowel or gut, fertility problems, polyps in the nose, clubbing of the finger/toe nails and skin, pain in the hands or feet, angiokeratoma, decreased perspiration, corneal and lenticular opacities, cataracts, mitral valve prolapse and/or regurgitation, cardiomegaly, temperature intolerance, difficulty walking, difficulty swallowing, progressive vision loss, progressive hearing loss, hypotonia, macroglossia, areflexia, lower back pain, sleep apnea, orthopnea, somnolence, lordosis, or scoliosis. It is understood that due to the diverse nature of the defective or absent proteins and the resulting disease phenotypes (e.g., symptomatic presentation of a metabolic disorder), a given disorder will generally present only symptoms characteristic to that particular disorder. For example, a patient with Fabry disease can present a particular subset of the above-mentioned symptoms such as, but not limited to, temperature intolerance, corneal whirling, pain, skin rashes, nausea, or diarrhea. A patient with Gaucher syndrome can

present with splenomegaly, cirrhosis, convulsions, hypertonia, apnea, osteoporosis, or skin discoloration.

In addition to the administration of one or more altered N-glycosylation molecules described herein, a metabolic disorder can also be treated by proper nutrition and vitamins (e.g., cofactor therapy), physical therapy, and pain medications.

Depending on the specific nature of a given metabolic disorder, a patient can present these symptoms at any age. In many cases, symptoms can present in childhood or in early adulthood. For example, symptoms of Fabry disease can present at an early age, e.g., at 10 or 11 years of age.

As used herein, a subject "at risk of developing a metabolic disorder" (such as one described herein) is a subject that has a predisposition to develop a disorder, i.e., a genetic predisposition to develop metabolic disorder as a result of a mutation in an enzyme such as alpha-L-iduronidase, beta-D-galactosidase, beta-glucosidase, beta-hexosaminidase, beta-D-mannosidase, alpha-L-fucosidase, arylsulfatase B, arylsulfatase A, alpha-N-acetylgalactosaminidase, aspartylglucosaminidase, iduronate-2-sulfatase, alpha-glucosaminidase-N-acetyltransferase, beta-D-glucuronidase, hyaluronidase, alpha-L-mannosidase, alpha-neuromimidine, phosphotransferase, acid lipase, acid ceramidase, sphingomyelinase, thioesterase, cathepsin K, or lipoprotein lipase. Clearly, subjects "at risk of developing a metabolic disorder" are not all the subjects within a species of interest.

A subject "suspected of having a disorder" is one having one or more symptoms of a disorder (e.g., a metabolic disorder or any other disorder described herein) such as any of those described herein.

#### (ii) Cancer

Cancer is a class of diseases or disorders characterized by uncontrolled division of cells and the ability of these to spread, either by direct growth into adjacent tissue through invasion, or by implantation into distant sites by metastasis (where cancer cells are transported through the bloodstream or lymphatic system). Cancer can affect people at all ages, but risk tends to increase with age. Types of cancers can include, e.g., lung cancer, breast cancer, colon cancer, pancreatic cancer, renal cancer, stomach cancer, liver cancer, bone cancer, hematological cancer, neural tissue cancer, melanoma, thyroid cancer, ovarian cancer, testicular cancer, prostate cancer, cervical cancer, vaginal cancer, or bladder cancer.

As used herein, a subject "at risk of developing a cancer" is a subject that has a predisposition to develop a cancer, i.e., a genetic predisposition to develop cancer such as a mutation in a tumor suppressor gene (e.g., mutation in BRCA1, p53, RB, or APC) or has been exposed to conditions that can result in cancer. Thus, a subject can also be one "at risk of developing a cancer" when the subject has been exposed to mutagenic or carcinogenic levels of certain compounds (e.g., carcinogenic compounds in cigarette smoke such as Acrolein, Arsenic, Benzene, Benz[a]anthracene, Benzo[a]pyrene, Polonium-210 (Radon), Urethane, or Vinyl Chloride). Moreover, the subject can be "at risk of developing a cancer" when the subject has been exposed to, e.g., large doses of ultraviolet light or X-irradiation, or exposed (e.g., infected) to a tumor-causing/associated virus such as papillomavirus, Epstein-Barr virus, hepatitis B virus, or human T-cell leukemia-lymphoma virus. From the above it will be clear that subjects "at risk of developing a cancer" are not all the subjects within a species of interest.

A subject "suspected of having a cancer" is one having one or more symptoms of a cancer. Symptoms of cancer are well-known to those of skill in the art and include, without limitation, breast lumps, nipple changes, breast cysts, breast

pain, weight loss, weakness, excessive fatigue, difficulty eating, loss of appetite, chronic cough, worsening breathlessness, coughing up blood, blood in the urine, blood in stool, nausea, vomiting, liver metastases, lung metastases, bone metastases, abdominal fullness, bloating, fluid in peritoneal cavity, vaginal bleeding, constipation, abdominal distension, perforation of colon, acute peritonitis (infection, fever, pain), pain, vomiting blood, heavy sweating, fever, high blood pressure, anemia, diarrhea, jaundice, dizziness, chills, muscle spasms, colon metastases, lung metastases, bladder metastases, liver metastases, bone metastases, kidney metastases, and pancreas metastases, difficulty swallowing, and the like.

In addition to the administration of one or more altered N-glycosylation molecules described herein, a cancer can also be treated by chemotherapeutic agents, ionizing radiation, immunotherapy agents, or hyperthermotherapy agents. Chemotherapeutic agents include, e.g., cisplatin, carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, adriamycin, ifosfamide, melphalan, chlorambucil, busulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide, verapamil, podophyllotoxin, tamoxifen, taxol, transplatin, 5-fluorouracil, vincristin, vinblastin, and methotrexate.

#### (iii) Inflammatory Disorders

An "inflammatory disorder," as used herein, refers to a process in which one or more substances (e.g., substances not naturally occurring in the subject), via the action of white blood cells (e.g., B cells, T cells, macrophages, monocytes, or dendritic cells) inappropriately trigger a pathological response, e.g., a pathological immune response. Accordingly, such cells involved in the inflammatory response are referred to as "inflammatory cells." The inappropriately triggered inflammatory response can be one where no foreign substance (e.g., an antigen, a virus, a bacterium, a fungus) is present in or on the subject. The inappropriately triggered response can be one where a self-component (e.g., a self-antigen) is targeted (e.g., an autoimmune disorder such as multiple sclerosis) by the inflammatory cells. The inappropriately triggered response can also be a response that is inappropriate in magnitude or duration, e.g., anaphylaxis. Thus, the inappropriately targeted response can be due to the presence of a microbial infection (e.g., viral, bacterial, or fungal). Types of inflammatory disorders (e.g., autoimmune disease) can include, but are not limited to, osteoarthritis, rheumatoid arthritis (RA), spondyloarthropathies, POEMS syndrome, Crohn's disease, multicentric Castleman's disease, systemic lupus erythematosus (SLE), multiple sclerosis (MS), muscular dystrophy (MD), insulin-dependent diabetes mellitus (IDDM), dermatomyositis, polymyositis, inflammatory neuropathies such as Guillain Barre syndrome, vasculitis such as Wegener's granulomatosis, polyarteritis nodosa, polymyalgia rheumatica, temporal arteritis, Sjogren's syndrome, Bechet's disease, Churg-Strauss syndrome, or Takayasu's arteritis. Also included in inflammatory disorders are certain types of allergies such as rhinitis, sinusitis, urticaria, hives, angioedema, atopic dermatitis, food allergies (e.g., a nut allergy), drug allergies (e.g., penicillin), insect allergies (e.g., allergy to a bee sting), or mastocytosis. Inflammatory disorders can also include ulcerative colitis and asthma.

A subject "at risk of developing an inflammatory disorder" refers to a subject with a family history of one or more inflammatory disorders (e.g., a genetic predisposition to one or more inflammatory disorders) or one exposed to one or more inflammation-inducing conditions. For example, a subject can have been exposed to a viral or bacterial superantigen such as, but not limited to, staphylococcal enterotoxins (SEs),

a *streptococcus pyogenes* exotoxin (SPE), a *staphylococcus aureus* toxic shock-syndrome toxin (TSST-1), a streptococcal mitogenic exotoxin (SME) and a streptococcal superantigen (SSA). From the above it will be clear that subjects "at risk of developing an inflammatory disorder" are not all the subjects within a species of interest.

A subject "suspected of having an inflammatory disorder" is one who presents with one or more symptoms of an inflammatory disorder. Symptoms of inflammatory disorders are well known in the art and include, but are not limited to, redness, swelling (e.g., swollen joints), joints that are warm to the touch, joint pain, stiffness, loss of joint function, fever, chills, fatigue, loss of energy, headaches, loss of appetite, muscle stiffness, insomnia, itchiness, stuffy nose, sneezing, coughing, one or more neurologic symptoms such as dizziness, seizures, or pain.

In addition to the administration of one or more altered N-glycosylation molecules described herein, an inflammatory disorder can also be treated by non-steroidal anti-inflammatory drug (NSAID), a disease-modifying anti-rheumatic drug (DMARD), a biological response modifier, or a corticosteroid. Biological response modifiers include, e.g., an anti-TNF agent (e.g., a soluble TNF receptor or an antibody specific for TNF such as adalimumab, infliximab, or etanercept).

Methods suitable for treating (e.g., preventing or ameliorating one or more symptoms of) any of the disorders described herein using any of the altered N-glycosylation molecules (or pharmaceutical compositions thereof) are set forth in the following section.

#### Pharmaceutical Compositions and Methods of Treatment

An altered N-glycosylation molecule (e.g., an altered N-glycosylation form of a target molecule such as a target protein) can be incorporated into a pharmaceutical composition containing a therapeutically effective amount of the molecule and one or more adjuvants, excipients, carriers, and/or diluents. Acceptable diluents, carriers and excipients typically do not adversely affect a recipient's homeostasis (e.g., electrolyte balance). Acceptable carriers include biocompatible, inert or bioabsorbable salts, buffering agents, oligo- or polysaccharides, polymers, viscosity-improving agents, preservatives and the like. One exemplary carrier is physiologic saline (0.15 M NaCl, pH 7.0 to 7.4). Another exemplary carrier is 50 mM sodium phosphate, 100 mM sodium chloride. Further details on techniques for formulation and administration of pharmaceutical compositions can be found in, e.g., Remington's Pharmaceutical Sciences (Maack Publishing Co., Easton, Pa.). Supplementary active compounds can also be incorporated into the compositions.

Administration of a pharmaceutical composition containing an altered N-glycosylation molecule can be systemic or local. Pharmaceutical compositions can be formulated such that they are suitable for parenteral and/or non-parenteral administration. Specific administration modalities include subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, intrathecal, oral, rectal, buccal, topical, nasal, ophthalmic, intra-articular, intra-arterial, sub-arachnoid, bronchial, lymphatic, vaginal, and intra-uterine administration.

Administration can be by periodic injections of a bolus of the pharmaceutical composition or can be uninterrupted or continuous by intravenous or intraperitoneal administration from a reservoir which is external (e.g., an IV bag) or internal (e.g., a bioerodable implant, a bioartificial organ, or a colony of implanted altered N-glycosylation molecule production cells). See, e.g., U.S. Pat. Nos. 4,407,957, 5,798,113, and 5,800,828, each incorporated herein by reference in their

entirety. Administration of a pharmaceutical composition can be achieved using suitable delivery means such as: a pump (see, e.g., Annals of Pharmacotherapy, 27:912 (1993); Cancer, 41:1270 (1993); Cancer Research, 44:1698 (1984), incorporated herein by reference in its entirety); microencapsulation (see, e.g., U.S. Pat. Nos. 4,352,883; 4,353,888; and 5,084,350, herein incorporated by reference in its entirety); continuous release polymer implants (see, e.g., Sabel, U.S. Pat. No. 4,883,666, incorporated herein by reference in its entirety); macroencapsulation (see, e.g., U.S. Pat. Nos. 5,284,761, 5,158,881, 4,976,859 and 4,968,733 and published PCT patent applications WO92/19195, WO 95/05452, the disclosures of each of which are incorporated herein by reference in their entirety); injection, either subcutaneously, intravenously, intra-arterially, intramuscularly, or to other suitable site; or oral administration, in capsule, liquid, tablet, pill, or prolonged release formulation.

Examples of parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, pump delivery, encapsulated cell delivery, liposomal delivery, needle-delivered injection, needle-less injection, nebulizer, aerosolizer, electroporation, and transdermal patch.

Formulations suitable for parenteral administration conveniently contain a sterile aqueous preparation of the altered N-glycosylation molecule, which preferably is isotonic with the blood of the recipient (e.g., physiological saline solution). Formulations can be presented in unit-dose or multi-dose form.

Formulations suitable for oral administration can be presented as discrete units such as capsules, cachets, tablets, or lozenges, each containing a predetermined amount of the altered N-glycosylation molecule; or a suspension in an aqueous liquor or a non-aqueous liquid, such as a syrup, an elixir, an emulsion, or a draught.

An altered N-glycosylation molecule (e.g., an altered N-glycosylation form of a target molecule such as a target protein) suitable for topical administration can be administered to a mammal (e.g., a human patient) as, e.g., a cream, a spray, a foam, a gel, an ointment, a salve, or a dry rub. A dry rub can be rehydrated at the site of administration. An altered N-glycosylation molecule can also be infused directly into (e.g., soaked into and dried) a bandage, gauze, or patch, which can then be applied topically. Altered N-glycosylation molecules can also be maintained in a semi-liquid, gelled, or fully-liquid state in a bandage, gauze, or patch for topical administration (see, e.g., U.S. Pat. No. 4,307,717, the content of which is incorporated herein by reference in its entirety).

Therapeutically effective amounts of a pharmaceutical composition can be administered to a subject in need thereof in a dosage regimen ascertainable by one of skill in the art. For example, a composition can be administered to the subject, e.g., systemically at a dosage from 0.01  $\mu\text{g/kg}$  to 10,000  $\mu\text{g/kg}$  body weight of the subject, per dose. In another example, the dosage is from 1  $\mu\text{g/kg}$  to 100  $\mu\text{g/kg}$  body weight of the subject, per dose. In another example, the dosage is from 1  $\mu\text{g/kg}$  to 30  $\mu\text{g/kg}$  body weight of the subject, per dose, e.g., from 3  $\mu\text{g/kg}$  to 10  $\mu\text{g/kg}$  body weight of the subject, per dose.

In order to optimize therapeutic efficacy, an altered N-glycosylation molecule can be first administered at different dosing regimens. The unit dose and regimen depend on factors that include, e.g., the species of mammal, its immune status, the body weight of the mammal. Typically, levels of an altered N-glycosylation molecule in a tissue can be monitored using appropriate screening assays as part of a clinical testing procedure, e.g., to determine the efficacy of a given treatment regimen.

The frequency of dosing for an altered N-glycosylation molecule is within the skills and clinical judgement of medical practitioners (e.g., doctors or nurses). Typically, the administration regime is established by clinical trials which may establish optimal administration parameters. However, the practitioner may vary such administration regimes according to the subject's age, health, weight, sex and medical status. The frequency of dosing can be varied depending on whether the treatment is prophylactic or therapeutic.

Toxicity and therapeutic efficacy of such altered N-glycosylation molecules (e.g., an altered N-glycosylation form of target molecules such as target proteins) or pharmaceutical compositions thereof can be determined by known pharmaceutical procedures in, for example, cell cultures or experimental animals. These procedures can be used, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Pharmaceutical compositions that exhibit high therapeutic indices are preferred. While pharmaceutical compositions that exhibit toxic side effects can be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to normal cells (e.g., non-target cells) and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in appropriate subjects (e.g., human patients). The dosage of such pharmaceutical compositions lies generally within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For a pharmaceutical composition used as described herein (e.g., for treating a metabolic disorder in a subject), the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the concentration of the pharmaceutical composition which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography.

As defined herein, a "therapeutically effective amount" of an altered N-glycosylation molecule is an amount of the molecule that is capable of producing a medically desirable result (e.g., amelioration of one or more symptoms of a metabolic disorder or decreased proliferation of cancer cells) in a treated subject. A therapeutically effective amount of an altered N-glycosylation molecule (i.e., an effective dosage) includes milligram or microgram amounts of the compound per kilogram of subject or sample weight (e.g., about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram).

The subject can be any mammal, e.g., a human (e.g., a human patient) or a non-human primate (e.g., chimpanzee, baboon, or monkey), a mouse, a rat, a rabbit, a guinea pig, a gerbil, a hamster, a horse, a type of livestock (e.g., cow, pig, sheep, or goat), a dog, a cat, or a whale.

An altered N-glycosylation molecule or pharmaceutical composition thereof described herein can be administered to a subject as a combination therapy with another treatment, e.g., a treatment for a metabolic disorder (e.g., a lysosomal

storage disorder). For example, the combination therapy can include administering to the subject (e.g., a human patient) one or more additional agents that provide a therapeutic benefit to the subject who has, or is at risk of developing, (or suspected of having) a metabolic disorder (e.g., a lysosomal storage disorder). Thus, the compound or pharmaceutical composition and the one or more additional agents are administered at the same time. Alternatively, the altered N-glycosylation molecule (e.g., protein or dolichol) can be administered first in time and the one or more additional agents administered second in time. The one or more additional agents can be administered first in time and the altered N-glycosylation molecule (e.g., protein or dolichol) administered second in time. The altered N-glycosylation molecule can replace or augment a previously or currently administered therapy. For example, upon treating with an altered N-glycosylation molecule of the invention, administration of the one or more additional agents can cease or diminish, e.g., be administered at lower levels. Administration of the previous therapy can also be maintained. In some instances, a previous therapy can be maintained until the level of the altered N-glycosylation molecule (e.g., the dosage or schedule) reaches a level sufficient to provide a therapeutic effect. The two therapies can be administered in combination.

It will be appreciated that in instances where a previous therapy is particularly toxic (e.g., a treatment for a metabolic disorder with significant side-effect profiles), administration of the altered N-glycosylation molecule (e.g., protein or dolichol) can be used to offset and/or lessen the amount of the previously therapy to a level sufficient to give the same or improved therapeutic benefit, but without the toxicity.

In some instances, when the subject is administered an altered N-glycosylation molecule (e.g., protein, dolichol, or a dolichol-linked lipid) or pharmaceutical composition of the invention the first therapy is halted. The subject can be monitored for a first pre-selected result, e.g., an improvement in one or more symptoms of a metabolic disorder such as any of those described herein (e.g., see above). In some cases, where the first pre-selected result is observed, treatment with the altered N-glycosylation molecule (e.g., an altered N-glycosylation protein or an altered N-glycosylation dolichol) is decreased or halted. The subject can then be monitored for a second pre-selected result after treatment with the altered N-glycosylation molecule (e.g., protein or dolichol) is halted, e.g., a worsening of a symptom of a metabolic disorder. When the second pre-selected result is observed, administration of the altered N-glycosylation molecule (e.g., protein or dolichol) to the subject can be reinstated or increased, or administration of the first therapy is reinstated, or the subject is administered both an altered N-glycosylation molecule (e.g., protein, dolichol, or a dolichol-linked lipid) and first therapy, or an increased amount of the altered N-glycosylation molecule (e.g., protein or dolichol) and the first therapeutic regimen.

The altered N-glycosylation molecule (e.g., protein or dolichol) can also be administered with a treatment for one or more symptoms of a disease (e.g., a metabolic disorder). For example, the altered N-glycosylation molecule (e.g., protein, dolichol, or a dolichol-linked lipid) can be co-administered (e.g., at the same time or by any combination regimen described above) with, e.g., a pain medication.

It is understood that in some embodiments, an altered N-glycosylation molecule is one in which the altered glycosylation increases the ability of the molecule to produce a medically relevant product. For example, an altered N-glycosylation molecule can be an enzyme capable of producing a therapeutic product (e.g., a small molecule or therapeutic

## 41

peptide), which enzyme's activity is increased or optimized by glycosylation. Such products and methods of using the products are within the scope of the present disclosure.

Any of the pharmaceutical compositions described herein can be included in a container, pack, or dispenser together with instructions for administration.

The following are examples of the practice of the invention. They are not to be construed as limiting the scope of the invention in any way.

## EXAMPLES

## Example 1

## Plasmids, Primers and Strains

Table 1 contains a list of all of the plasmids used in the construction of vectors (e.g., expression vectors) and deletion cassettes used in the experiments described herein. The MTLY60 strain of *Yarrowia lipolytica* was used in the experiments.

## 42

Table 2 contains a list of primers (the names of the primers) and the utility of the primers used in the following examples.

TABLE 1

Plasmids:
JMP62
pYLTsA
pYLHmL
pYLHmA
JMP113
JMP114
pRRQ2
JME 507
JME 509
JME 461
KS-LPR-URA3
KS-LPR-LEU2
Cre ARS68
LEU2

TABLE 2

Primers:	Name:	Use:
TCGCTATCAGTCTCTAGC (SEQ ID NO: 18)	Yloch1 prom fw	Amplification YLOCH1 Amplification YLOCH1 P fragment
TCTCTGTATACTTGTATGT ACTG (SEQ ID NO: 19)	Yloch1 ter rev	Amplification YLOCH1 Amplification YLOCH1 T fragment
CTAGGGATAACAGGGTAA TGGTGTGACGAAGTATCG AG (SEQ ID NO: 20)	YLOCH1 Pfrag rev	Amplification P fragment incl I-SceI site
CATTACCTGTATCCCTA GCGAGATCATGGACTGG (SEQ ID NO: 21)	YLOCH1 Tfrag fw	Amplification T fragment incl I-SceI site
GACGCGGCCGCATGAGCT TCAACATTCCCAAAC (SEQ ID NO: 22)	YlMNS1 ORF + Ter (Pfrag) S	Amplification of YlMNS1 P frag. (ORF + terminator)
CTAGGGATAACAGGGTAA TACAAAATTCAGAAATAA AAATACTTTACAG (SEQ ID NO: 23)	YlMNS1 ORF + Ter (Pfrag) AS	Amplification of YlMNS1 P frag. (ORF + termin.) + I-SceI
CATTACCTGTATCCCTA AGTAACATGAGTGCTATG AG (SEQ ID NO: 24)	YlMNS1 Tfrag S	Amplification of YlMNS1 T frag. (downstream terminator.) + I-SceI
CGCTTAATTAAATGCATGG AGGTATTGCTG (SEQ ID NO: 25)	YlMNS1 Tfrag AS	Amplification of YlMNS1 T frag. (downstream terminator.)
GGTGCTTCGACTATCAGTT TCGGAGGATTGGGTGATTC TTTTTATG (SEQ ID NO: 26)	ScMNS1 mut 269-273 S	ScMNS1 mutation primer to shift to mam. Golgi type mannase => proof of concept in Sc
CATAAAAGAATCACCCA ATCCTCCGAAATGATAGT CGAAGCACC (SEQ ID NO: 27)	ScMNS1 mut 269-273 AS	YlMNS1 mutation primer to shift to mam. Golgi type mannase => proof of concept in Sc
TGAGCGGCCGCTTTTCTAC TTCAGAGCTGGAG (SEQ ID NO: 28)	YlMNN9 P fw	YlMNN9 KO primer
GGCTTAATTAATTGGTAGT GATATAATGTAAACGC (SEQ ID NO: 29)	YlMNN9 T rv	YlMNN9 KO primer

TABLE 2-continued

Primers:	Name:	Use:
TAGGGATAACAGGGTAAT CACGACACATACTCATTCA AG (SEQ ID NO: 30)	YlMNN9 P rv	YlMNN9 KO primer
ATTACCTGTGTATCCCTAG AAGGAGATGTAGCGTAAG (SEQ ID NO: 31)	YlMNN9 T fw	YlMNN9 KO primer
TGATAAATAGCTTAGATAC CACAG (SEQ ID NO: 32)	LIP2 rv	Reverse primer used for sequencing
ACATACAACCACACACAT C (SEQ ID NO: 33)	5' hp4d	Forward primer used for sequencing
GGCGGATCCATGGTGCTGC ACCCGTTTC (SEQ ID NO: 34)	YlMNN4 BamHI fw	Forward primer for amplification of YlMNN4
GGCCCTAGGCTACTCAAAC TCCTCGCGAATC (SEQ ID NO: 35)	YlMNN4 AvrII rv	Reverse primer for amplification of YlMNN4
GGTCTCGCCAGCGGCCCA CCCTCTTC (SEQ ID NO: 36)	HAC1FW06-003	Forward primer region around HAC1 splice site
CTAGATCAGCAATAAAGT CGTGCCTGGGC (SEQ ID NO: 37)	HAC1RV06-001	Reverse primer region around HAC1 splice site
GGATCCATGTCTATCAAGC GAGAAGAG TCC (SEQ ID NO: 38)	HAC1FW06-002	Amplification of HAC1 gene includes start codon and BamHI restriction site
CCTAGGCTAGATCAGCAAT AAAGTCGTGCTGGGC (SEQ ID NO: 39)	HAC1RV06-006	Amplification of HAC1 gene includes stop codon and AvrII restriction site

## Example 2

*Yarrowia lipolytica* OCH1 and MNN9 Disruption

A strategy to knock out both OCH1 (GenBank® Accession No: AJ563920) and MNN9 (GenBank® Accession No: AF441127) genes in *Yarrowia lipolytica* was set up as described in Fickers et al. ((2003) J Microbiol Methods. 55(3):727-37) for the LIP2 gene. The gene construction strategy that was followed for the OCH1 gene is depicted in FIGS. 5A-5E.

The OCH1 KO fragment was isolated from the plasmid YIOCH1 PUT TOPO by restriction digest and by PCR and was transformed to *Yarrowia lipolytica* strain MTLY60. 20 uracil prototrophic strains were obtained and screened by PCR on genomic DNA (gDNA) using primers Yloch1 prom fw (SEQ ID NO:18) and Yloch1 ter rev (SEQ ID NO:19) to analyse the genomic integration of the plasmid. A fragment of the correct size (i.e., 2618 bp vs. 1894 bp in the wild type) was amplified in 2 of the 20 clones tested. Several clones contained a random integrated copy of the construct and therefore both fragments were amplified.

To remove the URA3 gene, the two positive clones were transformed with the episomal plasmid pRRQ2 that contains an expression cassette for the Cre recombinase. Removal of the URA3 gene was screened for by PCR on gDNA using primers Yloch1 prom fw and Yloch1 ter rev (see above). The 2328 bp fragment (incl. URA3) was absent from, and a 1075 bp (excl. URA3) fragment of 1075 bp was present in, the positive clones.

A Southern blot analysis was performed on the 2 positive clones to check whether aberrant DNA integration had occurred. Genomic DNA (gDNA) was double digested with EcoRV/HindIII, subjected to agarose-gel electrophoresis, and transferred to nitrocellulose membrane. The membrane was probed with a 500 bp SpeI/I-SceI fragment from plasmid YIOCH1 PT TOPO. A fragment of 1456 bp was present in Δoch1 PUT, whereas a fragment of 2066 bp in Δoch1 PT and a fragment of 2893 bp in the wild type strain was present.

A construction strategy to inactivate MNN9 was set up and is depicted in FIGS. 6A and 6B.

The disruption fragment was cut out of plasmid YlMNN9PUT TOPO by a NotI/PacI double digest and transformed to MTLY60 and Δoch1 PT clone 9. Several URA3 positive clones were obtained for both strains and they were screened for correct integration of the construct by PCR on gDNA after single clones were isolated. A fragment of 2349 bp was amplified in the disruptant strains, whereas in the non-transformants, a fragment of 2056 bp was amplified using primers YlMNN9 P fw and YlMNN9 T rv. (Table 2).

To analyze the N-glycan structures that were synthesized by the mutant strains, DSA-FACE was performed on glycans derived from mannoproteins (FIG. 7). The wild-type (MTLY60) strain has as main core type glycan structures mainly Man<sub>8</sub>GlcNAc<sub>2</sub> (structural formula I; FIG. 4A) and a substantial amount of Man<sub>9</sub>GlcNAc<sub>2</sub> (structural formula II; FIG. 4A) the latter most probably containing an additional mannose as a result of Och1p activity. Furthermore, some larger structures can be seen. The Δoch1 strain has mainly Man<sub>8</sub>GlcNAc<sub>2</sub> (structure formula I) and a small portion of

Man<sub>5</sub>GlcNAc<sub>2</sub> (structural formula II; FIG. 4A), both of which are sensitive to  $\alpha$ -1,2-mannosidase treatment (indicated  $\Delta$ och1  $\alpha$ -1,2-man) resulting in trimming to Man<sub>5</sub>GlcNAc<sub>2</sub> (structural formula IV; FIG. 4A). The  $\Delta$ mnn9 strain accumulates more Man<sub>5</sub>GlcNAc<sub>2</sub> (structural formula II; FIG. 4A) than the  $\Delta$ och1 strain, which indicates that Mnn9p is involved in the elongation of the glycan structure subsequent to Och1p activity. The double mutant  $\Delta$ och1  $\Delta$ mnn9 displays a glycosylation phenotype that resembles the one from the  $\Delta$ och1 strain.

### Example 3

#### Mutagenesis of MNS1

MNS1 (ER  $\alpha$ -1,2-mannosidase) is involved in the trimming of the Man<sub>5</sub>GlcNAc<sub>2</sub> to Man<sub>8</sub>GlcNAc<sub>2</sub> and has a strict substrate specificity in the sense that it is only able to trim the  $\alpha$ -1,2-mannose that is linked to the  $\alpha$ -1,3-mannose of the central arm (FIG. 2). To determine where the MNS1 gene could be mutagenized in order to shift its substrate specificity towards a Golgi type  $\alpha$ -1,2-mannosidase, the primary sequences of several ER type mannosidases were compared with Golgi type mannosidases. One region that is different between the two classes was identified. In addition, an oligosaccharide that was crystallised in the catalytic site of the Golgi type mannosidase into the yeast MNS1 was also analyzed to identify possible interactions between sugar and protein. Surprisingly, the same sites were identified using both methods.

The MNS1 gene from *Saccharomyces cerevisiae* (GenBank® Accession No: Z49631, sgd: YJR131W) was mutated in order to change its substrate specificity. Three mutated versions were made: two with one mutation (R273L and R273G) and one with 3 mutations (R269S/S272G/R273L) in the same region:

A) R273L (arginine 273 to leucine)

B) R273G (arginine 273 to glycine)

C) R269S/S272G/R273L (arginine 269 to serine/serine 272 to glycine/arginine 273 to leucine).

All mutations were made using the Quick Change (Stratagene) mutagenesis kit. Constructs were made to express the 3 different mutant genes under control of the strong constitutive TPI1 promoter. Oligonucleotides CGACTATCCGGTTCGGATCATGGGTGATCTTTTATGAG (SEQ ID NO:40) and CTCATAAAAAGAATCACCCAATGATCGAACC GGATAGTCG (SEQ ID NO:41) were used to generate mutant R273L, and oligonucleotides CGACTATCCGGTTCGGATCAGGTGGTGATTCTTTTATGAG (SEQ ID NO:42) and CTCATAAAAAGAATCACCACTGATCGAACC GGATAGTCG (SEQ ID NO:43) were used to obtain mutant R273G using the wild type gene as a template. Oligonucleotides GGTGCTTCGACTATCAGTTTCGGAGGATTGGGTGATTCTTTTATG (SEQ ID NO:44) and CATAAAAAGAATCACCCAATCCTCCGAACTGATGTCGAAGCACC (SEQ ID NO:45) were used to obtain mutant R269S/S272G/R273L using mutant R273L as template DNA. Via PCR reaction using oligonucleotides CCCGATATCGGATCCATGAAGAACTCTGTCTGGTATTTC (SEQ ID NO:46) and GGGAAGCTTAACGCGGTTC-CAGCGGGTCCGGATACGGCACCGGCGCACCCAA-CGAC CAACCTGTGGTCAG (SEQ ID NO:47) the coding sequence of an E-tag was added at the 3' end of the mutant and the wild type MNS1 open reading frames to allow protein detection after expression. An overview of the construction strategy is presented in FIGS. 8A-8E.

The three constructs, as well as the non-mutated gene (as a negative control), were transformed to *S. cerevisiae* strain XW27 (MAT $\alpha$  leu2 ura3 trp1 his3 ade2 lys2 och1::LEU2 mnn1::URA3 mnn6::ADE2) using TRP1 as a selection marker after digestion of the plasmids with XbaI to direct the construct to the TRP1 locus in the *S. cerevisiae* genome. The latter strain is able to synthesize uniform Man<sub>8</sub>GlcNAc<sub>2</sub> (on its glycoproteins. If the mutated enzyme is active this Man<sub>8</sub>GlcNAc<sub>2</sub> (structural formula I; FIG. 4A) should be trimmed to Man<sub>5</sub>GlcNAc<sub>2</sub> (structural formula IV; FIG. 4A), Man<sub>6</sub>GlcNAc<sub>2</sub> (structural formula V; FIG. 4A) and/or Man<sub>7</sub>GlcNAc<sub>2</sub> (structural formula VI; FIG. 4A).

Tryptophan prototrophic strains were isolated, grown in liquid SDC-trp medium and mannoproteins were prepared. N-glycans derived from mannoproteins were analysed via DSA-FACE. As can be appreciated from FIG. 9, a small amount of Man<sub>8</sub>GlcNAc<sub>2</sub> (structural formula I; FIG. 4A) from the strains that contain the R273G and R269S/S272G/R273L mutations are converted to Man<sub>5</sub>GlcNAc<sub>2</sub> (structural formula IV; FIG. 4A), Man<sub>6</sub>GlcNAc<sub>2</sub> (structural formula V; FIG. 4A) and Man<sub>7</sub>GlcNAc<sub>2</sub> (structural formula VI; FIG. 4A). The expression of the other mutant or the wild type gene cause an altered N-glycosylation phenotype. To evaluate whether all mutants are equally well expressed, a Western blot analysis was performed using an antibody specific for an E-tag (a 13 amino acid epitope added to the MNS1 proteins). All mutant proteins, as well as the wild-type MNS1 protein, were expressed equally well.

### Example 4

#### Increasing Phosphorylation

##### Expression of *Yarrowia lipolytica* MNN4

To increase the phosphorylation of Man<sub>8</sub>GlcNAc<sub>2</sub>, *Yarrowia lipolytica* MNN4 (a homologue of the *P. pastoris* PNO1) was overexpressed in *Yarrowia lipolytica* to promote the core type phosphorylation of N-glycans.

The coding sequence of the *Yarrowia lipolytica* MNN4 (XM\_503217, YALI0D24101g) gene was amplified using primers GGCGGATCCATGGTGCTGCACCCGTTTC (YIMNN4 BamHI fw; SEQ ID NO:34) and GGCCCTAGGCTACTCAAACCTCTCGCGAATC (YIMNN4 AvrII rv; SEQ ID NO:35). This open reading frame (ORF) was cloned into the plasmid using BamHI and AvrII sites, which placed the ORF under control of the hp4d promoter of plasmid pYIHURA3 that contains the URA3d1 gene as a selection marker and the zeta sequences for improving random integration (FIG. 10).

Prior to transformation in the MTLY60  $\Delta$ och1 strain, the plasmid containing the MNN4 expression cassette was digested either with Eco47III for integration in the URA3 locus, PvuI for integration in the MNN4 locus, or RsrII/BstBI for random integration. Transformants targeted to the URA3 and MNN4 locus were analysed by PCR using a primer in the hp4d promoter and one in the LIP2 terminator. Transformants with random integration of the construct were evaluated by Southern blot analysis.

To evaluate whether manno-phosphorylation was increased we analysed N-glycans derived from secreted glycoproteins after 48 hours culture in YPD medium by DSA-FACE capillary electrophoresis (FIG. 11). The amount of Man<sub>8</sub>GlcNAc<sub>2</sub> (structural formula I) was drastically reduced in favour of two structures that migrate faster (compared to Man<sub>8</sub>GlcNAc<sub>2</sub> (structural formula I; FIG. 4A)) and that are likely to contain one (P) (structural formula X or XI; FIG. 4B)

and two (PP) (structural formula XII; FIG. 4B) phosphate residues, respectively (FIG. 11). Thus, it can be concluded that the random integrated expression cassettes perform better than the cassettes integrated in the URA3 locus or the MNN4 locus, in that order. The MZ2 exhibited the highest level of phosphorylation.

Assuming that both peaks derive from the  $\text{Man}_8\text{GlcNAc}_2$  (structural formula I; FIG. 4A) peak, the amount of  $\text{Man}_8\text{GlcNAc}_2$  converted to phosphorylated glycans was quantitated (Table 3).

TABLE 3

N-glycan struct.	height	area	% signal	Phosph-Status
<b>Strain Aoch1</b>				
M82P (struct. form. XII)	18	302	1.02826	18.91045*
M8P (struct. form. X or XI)	261	5252	17.88219	
M8 (struct. form. I)	928	23816	81.08955	81.08955*
		29370	100	100
<b>Strain MU5</b>				
M82P (struct. form. XII)	1319	19736	27.16773	81.17283*
M8P (struct. form. X or XI)	2025	39232	54.00509	
M8 (struct. form. I)	539	13677	18.82717	18.82717*
		72645	100	100
<b>Strain MZ2</b>				
M82P (struct. form. XII)	1182	17662	27.75299	83.11282*
M8P (struct. form. X or XI)	1803	35231	55.35984	
M8 (struct. form. I)	419	10747	16.88718	16.88718*
		63640	100	100

Table 3 Legend:

Height and area refer to the peak height and peak area as determined from electropherograms.

"% signal" refers to the proportion of each glycan in the N-glycan mixture.

The numbers identified by asterisk depict the proportion of phosphorylated  $\text{Man}_8\text{Gn}_2$  (top) and the proportion of non-phosphorylated  $\text{Man}_8\text{Gn}_2$  (bottom).

These results indicated that more than 80% of  $\text{Man}_8\text{GlcNAc}_2$  (structural formula I; FIG. 4A) that is present in the parent Aoch1 is phosphorylated in the strain that over expresses the YIMNN4 gene.

### Example 5

#### Modifying Glycosylation by Lipid-Linked Oligosaccharide Modification in the Endoplasmic Reticulum

#### Materials and Methods

#### Strains, Culture Conditions and Reagents.

*Escherichia coli* strains MC1061 or TOP10 or DH5 $\alpha$  were used for the amplification of recombinant plasmid DNA and grown in a thermal shaker at 37° C. in Luria-Broth (LB) medium supplemented with 100  $\mu\text{g}/\text{ml}$  of carbenicillin or 50  $\mu\text{g}/\text{ml}$  of kanamycin depending on the plasmids used.

*Yarrowia lipolytica* MTLY60 (ura3 leu2) strain was used as parent strain. All yeast strains were cultured in a 28° C. incubator. They were grown on YPD medium (2% dextrose, 2% bacto-peptone and 1% yeast extract) or synthetic dextrose complete (SDC) medium (0.17% YNB w/o amino acids and without ammonium sulphate, 1% glucose, 0.5%  $\text{NH}_4\text{Cl}$ , 50 mM K/Na phosphate buffer pH 6.8 and 0.077% Complete Supplement Mixture (Qbiogene Inc, Morgan Irvine, Calif.)). For selection of Ura+ and Leu+ transformants 0.077% CSM-ura or CSM-leu was added respectively.

#### Standard Genetic Techniques.

Transformation competent cells of *Yarrowia lipolytica* were prepared as described in Boisrame et al. (1996) J. Biol. Chem. 271(20):11668-75, the disclosure of which is incorporated herein by reference in its entirety. Genomic DNA from all yeast strains was isolated using a published protocol (Epicenter Kit catalogue No. MPY80200; Epicenter Biotechnologies, Madison, Wis.). The protocol involves non-enzymatic cell lysis at 65° C., followed by removal of protein by precipitation and nucleic acid precipitation and resuspension. PCR amplification was performed in a final volume of 50  $\mu\text{l}$  containing 5  $\mu\text{l}$  of 10 $\times$  buffer (200 mM Tris-HCl pH8.4 and 500 mM KCl), a variable quantity of  $\text{MgCl}_2$ , 2.5  $\mu\text{M}$  dNTP, 50 ng of template, 50 pmol of the proper primers and 2.5 units of either Taq or Pfu DNA polymerase. Cycling conditions used were as follows: denaturation at 94° C. for 10 minutes followed by hot start and 30 cycles of 94° C. for 45 seconds, suitable annealing temperature for 45 seconds and extension at 72° C. for 1 minute per kb followed by 10 min of extension at 72° C. DNA fragments (PCR products or fragments) recovered from gel were purified using NucleoSpin extract II (Macherey-Nagel). DNA sequencing was performed by VIB Genetic Service Facility (Antwerp, Belgium). Vector Construction.

(i) Knock-out (gene-replacement) of the ALG3 gene. The promoter fragment (P) of the ALG3 gene (GenBank® Accession No: XM\_503488, Genolevures: YALI0E03190g) was amplified from genomic DNA of the *Yarrowia lipolytica* MTLY60 strain by PCR with 5'CAGTGC GGCCGCACTC-CCTCTTTTCACTCACTATTG3' (SEQ ID NO:48) and 5'CATTACCCTGTTATCCCTACGCTCAGATCCAATTG-TTTTGGTGGTC3' (SEQ ID NO:49) as the forward and reverse primers, respectively, using Taq polymerase (Invitrogen). The overhanging A nucleotide was removed with T4 DNA polymerase (Fermentas, Ontario, Canada). The terminator fragment (T) of the ALG3 gene was amplified from genomic DNA of the *Yarrowia lipolytica* MTLY60 strain by PCR with 5'GTAGGGATAACAGGGTAATGCTCTCAAG-GACGGACCAGATGAGACTGTTATCG3' (SEQ ID NO:50) and 5'GACTTTAATTAAACCCCTATGTGGCACCT-CAACCCACATCTCCCGTC3' (SEQ ID NO:51) as the forward and reverse primers, respectively, using the proofreading Pfu DNA polymerase (Fermentas). Because of overlapping primer sequences containing an IScel restriction site, both fragments could be linked by PCR with the P-forward primer and the T-reverse primer. This co-amplicon was then subcloned in a pCR-2.1 TOPO TA (Invitrogen) vector and the correctness of the co-amplicon's sequence was confirmed by sequencing. The co-amplicon was then cloned using the NotI-PacI sites into an intermediate vector.

(ii) Overexpression of the ALG6 gene. The ALG6 ORF (1725 bp) together with the terminator (415 bp downstream) of the ALG6 gene (GenBank® Accession No: XM\_502922, Genolevures: YALI0D17028g) were cloned from genomic DNA of the *Yarrowia lipolytica* MTLY60 strain by PCR with 5'CAGTGGATCCATGAACCTCTCCTATTTTCACTACCG-3' (SEQ ID NO:52) and 5'GACTCCTAGGAAGCTTCAG-GTTACAAAGTTGTTAC3' (SEQ ID NO:53) as the forward and reverse primers, respectively, using the proofreading Pfu DNA polymerase (Fermentas). The sequence was cloned in pCR-Blunt II-TOPO (Invitrogen) and the correctness of the ALG6 ORF sequence was confirmed by sequencing (as above). Next, the ALG6 ORF was cloned in a vector (pYL-HmA) containing the hp4d promoter via BamHI and AvrII and subsequently cloned in the intermediate vector via the unique restriction sites ClaI and HindIII present in the terminator fragment of ALG3.

(iii) Selection marker cassette. To remove the selectable marker URA3 from the host genomic DNA, the Cre-lox recombination system was used, e.g., as described by Fickers et al. ((2003) J. Microbiol. Methods 55(3):727-737, the disclosure of which is incorporated herein by reference in its entirety). Upon expression of the Cre recombinase from the plasmid pRRQ2 (hp4d-cre, LEU2) (a gift from the Institut National de Recherche Agronomique (INRA)), the marker gets excised by recombination between the two lox sites. In both constructs, with and without the ALG6 overexpression cassette, the URA3 selection marker flanked by lox sites, was inserted in the introduced I-SceI site between P and T fragments of the vector, resulting in a "PUT" construct.

Preparation of Mannoproteins.

Yeast strains were grown overnight in 10 ml standard YPD medium in 50 ml falcon tubes, rotating at 250 rpm in a 28° C. incubator. The cells were then pelleted by centrifugation at 4000 rpm at 4° C. The supernatants were removed, and the cells were first washed with 2 ml of 0.9% NaCl solution followed by two washes with 2 ml of water and subsequently resuspended in 1.5 ml of 0.02 M sodium citrate pH 7 in a microcentrifuge tube. After autoclaving the tubes for 90 minutes at 121° C., the tubes were vortexed and the cellular debris was pelleted by centrifugation. The supernatants were collected and the mannoproteins were precipitated overnight with 4 volumes of methanol at 4° C. with rotary motion. The precipitate was then obtained by centrifugation of the alcohol precipitated material. The pellets were allowed to dry and dissolved in 50 µl of water.

#### Sugar Analysis.

DNA sequencer-assisted (DSA), fluorophore-assisted carbohydrate electrophoresis (FACE) was performed with an ABI 3130 DNA sequencer as described by Callewaert et al. (2001; supra). Briefly, glycoproteins were denatured for 1 hour in RCM buffer (8M urea, 360 mM Tris pH 8.6 and 3.2 mM EDTA) at 50° C. followed by immobilization on a pretreated PVDF membrane of a IP plate containing 15 µl RCM. Prewetting of the membrane was done with 300 µl MeOH, 3 times washed with 300 µl water and 50 µl RCM, followed by vacuum removal. The glycoproteins were reduced for 1 hour with 50 µl 0.1M dithiothreitol and washed 3 times with 300 µl water. A 30 minute incubation in the dark with 50 µl 0.1M iodoacetic acid was used to carboxymethylate the SH groups, followed by 3 washes with 300 µl water. The plates were subsequently incubated for 1 hour with 100 µl 1% polyvinylpyrrolidone 360 to saturate the unoccupied binding sites on the membrane, again followed by 3 washes with 300 µl water. Next, the N-glycans were released by 3 hours treatment with peptide: N-glycosidase F (PNGase F)×U in 50 µl of 10 mM Tris-acetate pH 8.3. N-glycans were recuperated and derivatized with the fluorophore 8-aminopyrene-1,3,6-trisulfonate (APTS) by reductive amination. This was accomplished with an overnight (ON) incubation at 37° C. with 1 µl of 1:1 mixture of 20 mM APTS in 1.2M citric acid and 1M NaCNBH<sub>3</sub> in DMSO and quenching by addition of 4 µl water. Excess label was removed by size fractionation on Sephadex G-10 resin. The remaining labeled N-glycans were then concentrated by evaporation. The N-glycans of RNase B and an oligomaltose ladder were included as size markers. Data analysis was performed using Genemapper® software (Applied Biosystems). Glycosidase digests on the labeled sugars were performed ON at 37° C. in 100 mM NH<sub>4</sub>AC pH 5. Additional Jack bean (JB) mannosidase was added after ON digestion and left for another 24 hours at 37° C.

#### Disruption of the ALG3 gene in *Yarrowia lipolytica*

To disrupt the ALG3 gene, a vector was generated that includes parts of the promoter and terminator of ALG3 and

has a URA3 selection marker cassette and was designated pYLa1g3PUT. A NotI and PacI site were integrated to linearize the vector and thereby remove the *E. coli* related DNA elements. Double homologous recombination at the promoter and terminator site was used to replace ALG3 with the URA3 selectable marker, which resulted in an alg3::URA3 mutant strain. The knockout strategy applied was described by Fickers et al. (2003; supra) and makes use of the Cre-lox recombination system, that facilitates efficient marker rescue. Upon integration in the genomic ALG3 contig the Alg3p  $\alpha$ -1,6-mannosyltransferase activity should be lost. This was monitored by analyzing the glycosylation pattern of the mannoproteins of several transformants. The N-glycans derived from mannoproteins were analysed by DSA-FACE (capillary electrophoresis) and treated with a selection of exoglycosidases to reveal the structures. Seven out of 24 transformants gave a change in glycosylation profile (three of which are depicted in FIG. 13). In all seven transformants, correct integration of the knockout cassette in the genome could be confirmed by PCR. Three main glycan structures were found by analyzing the profiles: (i) one (structural formula VII; FIG. 4A) that runs at the same size as the Man<sub>5</sub>GlcNAc<sub>2</sub> structure of RNase B (the latter being structural formula IV; FIG. 4A); (ii) one at a distance of one glucose-unit extra; and (iii) one at the distance of two extra glucose-units. (FIG. 13). These results indicate that ALG3 was disrupted in these cells.

#### Overexpression of $\alpha$ -1,6-mannosyltransferase Alg6p

A strategy was developed in which a constitutively active overexpression cassette for the first glucosyltransferase, i.e., Alg6p, was incorporated into the alg3 gene replacement vector. This vector was designated pYLa1g3PUT-ALG6. A NotI/PacI fragment of this vector was transformed into the *Yarrowia lipolytica* MTLY60 strain. In this way, disruption of ALG3 and overexpression of ALG6 under control of the hp4d promoter is achieved. Correct integration in the genome was again confirmed by PCR. DSA-FACE analysis of the N-glycans derived from mannoproteins showed that half of the transformants, i.e., 12 out of 24, exhibited a change in glycosylation pattern comparing to the WT strain. Overexpression of ALG6 led to a mild clonal variation (FIG. 13).

#### Identification of the N-Glycan Structures

To further elucidate the nature of the glycan structures from the experiments described above, in vitro digests of glycans derived from the mannoproteins (as above) were performed with a selection of exoglycosidases. The mannoprotein glycans were analyzed with the following enzymes:  $\alpha$ -1,2-mannosidase;  $\alpha$ -mannosidase (JB) and glucosidase II. Three observed glycan structures represent Man<sub>5</sub>GlcNAc<sub>2</sub> (structural formula VII; FIG. 4A), GlcMan<sub>5</sub>GlcNAc<sub>2</sub> (structural formula VIII; FIG. 4B) and Glc<sub>2</sub>Man<sub>5</sub>GlcNAc<sub>2</sub> (structural formula IX; FIG. 4B) (FIG. 14). These results indicate that there is very little to no high mannose elongation by  $\alpha$ -1,6-mannosyltransferases (e.g., Och1p).

To determine if ALG6 overexpression is necessary for promoting N-glycosylation site-occupancy, Lipase 2 (LIP2) from *Yarrowia lipolytica* was expressed in three different strains of *Yarrowia*: MTLY60, MTLY60 $\Delta$ alg3 and MTLY60 $\Delta$ alg3ALG6. A construct for the *Yarrowia lipolytica* LIP2, under control of a TEF constitutive promoter was obtained from INRA. The expression cassette was transformed to the above-mentioned strains and the expression of the protein was verified by subjecting the supernatant prepared from the transformed cells to SDS-PAGE analysis

(FIG. 28). The Lip2p protein has 2 glycosylation sites. Lip2p protein derived from the *alg3*-deficient ("knockout") yeast strain was resolved by SDS-PAGE into three distinct bands that were visualized using Coomassie blue staining of the gel (FIG. 28). To confirm that all three forms of protein in the gel were different glycosylation forms of the Lip2p protein, Lip2p protein obtained from the *alg3*-deficient ("knockout") yeast strain was subject to treatment with PNGase F (an enzyme that removes oligosaccharide residues from glycoproteins) and then subjected to SDS-PAGE analysis as described above. Treatment of the Lip2p protein with PNGase F resulted in a single band (which had the same molecular weight as non-glycosylated Lip2p) on the gel following Coomassie blue staining and indicated that all three forms of protein previously observed were different glycosylation forms of the same Lip2p molecule. The same is true for the Lip2p derived from the *alg3*ALG6 strain. However, the amount of protein in a reduced glycosylation form is decreased. Thus, it can be concluded that overexpression of ALG6 can (at least partially) restore N-glycosylation site-occupancy, which is reduced in the *alg3* knockout mutant yeast strain.

#### Removing Capping Glucose Structures

Next, to eliminate mono (structural formula VIII; FIG. 4B) and bi-glucosylated (structural formula IX; FIG. 4B)  $\text{Man}_5\text{GlcNAc}_2$  (structural formula VII; FIG. 4B) structures in vivo, cells were genetically engineered to overexpress the  $\alpha$ -subunit of the enzyme glucosidase II. The  $\alpha$  subunit of glucosidase II of *Yarrowia* (GenBank® Accession No: XM\_500574) and the  $\alpha$  subunit of glucosidase II *Trypanosoma brucei* (GenBank® Accession No: AJ865333) were independently cloned as two strategies to overexpress the protein. The  $\alpha$  subunit of glucosidase II *Trypanosoma brucei* was chosen since its natural substrate is  $\text{GlcMan}_5\text{GlcNAc}_2$  (structural formula VIII; FIG. 4B). Both genes were cloned under control of the constitutive *hp4d* promoter and their plasmids contain the URA3 marker. These constructs were transformed into *alg3* mutant yeast strains, both with and without ALG6 overexpression.

Oligosaccharides were prepared from secreted proteins derived from cultured cells containing the constructs and the profile of the oligosaccharides was determined by DSA-FACE analysis. All transformants gave the same DSA-FACE profile, two different clones of each glucosidaseII  $\alpha$  are depicted in FIG. 29. From these results it was concluded that the overexpression of either the *Yarrowia* or the *Trypanosoma* glucosidase II  $\alpha$  subunit has only a minor effect on the amount of mono (structural formula VIII; FIG. 4B) and bi-glucosylated (structural formula IX; FIG. 4B)  $\text{Man}_5\text{GlcNAc}_2$  (structural formula VII; FIG. 4A) structures.

#### Expression of Glucosidase II $\alpha$ -Subunits of *Yarrowia lipolytica* and *Trypanosoma brucei* Tagged with an HDEL Sequence

To improve the effect of the expression of *Yarrowia* or the *Trypanosoma* glucosidase II  $\alpha$  subunit on removing glucose residues from  $\text{Man}_5\text{GlcNAc}_2$  in vivo, a nucleic acid encoding an HDEL tag was added using molecular biology techniques in frame to the 3' end of the nucleic acid encoding each of the two GlcII  $\alpha$  enzymes. The HDEL tag was meant to serve as a retrieval mechanism from the Golgi to the ER. Plasmids encoding HDEL-tagged glucosidase II sequences from both *Yarrowia lipolytica* (Y.I.) and *Trypanosoma brucei* (T.b.) under control of the *hp4d* promoter were transformed to the

*alg3* KO strain with and without overexpression of the ALG6 gene. As can be seen in FIG. 30, overexpression of the *Yarrowia lipolytica* glucosidase II  $\alpha$  subunit had only a minor effect on the amount of glucosylated structures. In contrast, overexpressing the  $\alpha$ -Glucosidase II of *Trypanosoma brucei*  $\alpha$  subunit with an extra HDEL tag leads to a reduction of the mono-glucose peak (see FIG. 31).

#### Treatment of Glucosylated Glycans with Mutanase

The above-described results demonstrate one exemplary means of reducing mono-glucosylated forms of  $\text{Man}_5\text{GlcNAc}_2$ . To reduce bi-glucosylated forms of  $\text{Man}_5\text{GlcNAc}_2$  from glycoproteins, the mutanase of *T. harzianum* was investigated as one potential solution. An enzyme preparation was obtained from Novozymes (Novozyme 234; Bagsvaerd, Denmark) and was used to digest oligosaccharides in vitro. That is, mutanase was added in different concentrations to the oligosaccharides derived from a *alg3*ALG6 strain (glycans:  $\text{Man}_5\text{GlcNAc}_2$ ,  $\text{GlcMan}_5\text{GlcNAc}_2$  and  $\text{Glc}_2\text{Man}_5\text{GlcNAc}_2$ ). As shown in the DSA-FACE profile of FIG. 32, the bi-glucose peak observed in the oligosaccharides was effectively reduced.

Next, the mutanase of *T. harzianum* was overexpressed in vivo. An HDEL-sequence containing mutanase was synthesized as a codon-optimized cDNA for expression in *Yarrowia lipolytica*. The mature protein was cloned in frame with the *LIP2* pre signal sequence under control of the TEFL promoter (FIG. 33). This construct is transformed into *alg3* mutant yeast strains, both with and without ALG6 overexpression. Oligosaccharides are prepared from cultured cells containing the construct and the profile of the oligosaccharides is determined by DSA-FACE analysis. It is expected that the DSA-FACE profile will show a reduction in the bi-glucose peak observed in the oligosaccharides. From these results it will be concluded that the overexpression of mutanase in vivo is effective at reducing the bi-glucose peak observed in oligosaccharides as compared to cells not overexpressing the mutanase.

#### Co-Expression of YI GlcII $\alpha$ - and $\beta$ Subunits

It is known that the  $\alpha$ - and  $\beta$ -subunits of glucosidase II form a heterodimeric complex whereby the  $\beta$ -subunit is responsible for retrieval of the complex to the ER and is also involved in substrate recognition, whereas the  $\alpha$ -subunit contains the catalytic activity. Since the overexpression of only the  $\alpha$ -subunit of glucosidase II had a small effect on bi-glucose oligosaccharide structures, the  $\alpha$ - and  $\beta$ -subunits were co-expressed.

The open reading frame of the  $\beta$ -subunit (YALI0B03652g) was amplified from genomic DNA that was isolated from the MTLY60 strain using PCR and was cloned under control of the TEF1 and *hp4d* promoter. The constructs were made with LEU2 as a selection marker and with the glucosidase II  $\beta$ -subunit under control of the TEF1 and the *hp4d* promoter. These were transformed to the *alg3* knockout strains with and without ALG6 overexpression and overexpressing the *Yarrowia lipolytica* Glucosidase II  $\alpha$  subunit with and without an HDEL sequence tag. N-glycans were prepared from proteins secreted from the cells and the DSA-FACE profiles of the N-glycans are depicted in FIGS. 33 and 34 (*alg3* knockout with overexpression of ALG6). It can be concluded from these profiles that overexpressing the  $\beta$  subunit of glucosidase II from *Yarrowia lipolytica* did have a positive effect on the trimming of the glucosylated sugars. In general, the efficacy of the  $\beta$  subunit of glucosidase II was improved when

expressed under the TEF1 promoter. The glucosylated structures were even more reduced when the *Yarrowia lipolytica* glucosidase II  $\alpha$  subunit contained an HDEL tag (FIGS. 33 and 34).

For alg3-deficient cells without ALG6 overexpression, similar results regarding reduction of glucosylated structures were observed for each of the different cell populations (FIG. 35).

#### Expression of *Aspergillus* GlsII a and b Subunit

In order for the glucose residues to be removed from the glucose bearing structures that occur in alg3-deficient background, the *Aspergillus niger* mature (lacking signal peptide) glucosidase II  $\alpha$  and  $\beta$  were synthesized as codon-optimized cDNA for expression in *Yarrowia lipolytica* ( $\alpha$ -subunit (SEQ ID NO:7; FIGS. 36A-36B)  $\beta$ -subunit: (SEQ ID NO:8; FIG. 37). *Aspergillus niger* (An) glucosidase  $\alpha$  subunit was cloned under control of the constitutive TEF1 and hp4d promoters and had URA3 gene as a selection marker. The expression cassettes (ORFs under control of TEF1 and hp4d) were transformed to *Yarrowia lipolytica* alg3ALG6 strain. Transformant candidates were grown in YPD and glycans from secreted proteins were analysed by DSA-FACE. It can be deduced from FIG. 38 that the two glucosylated structures are less abundant in the transformant strains compared to the non-transformant (alg3ALG6).

To further reduce the glucosylated glycan structures a construct is made with  $\beta$ -subunit of the *Aspergillus niger* glucosidase II under control of TEF1 promoter or hp4d promoter with LEU2 as a selection marker. This construct is transformed to *Yarrowia lipolytica* alg3ALG6 strain expressing the An glucosidase II  $\alpha$ -subunit. It is expected that expression of the  $\beta$ -subunit of the *Aspergillus niger* glucosidase II will result in a decrease in glucosylated structures in *Yarrowia lipolytica* cells.

#### Example 6

##### Identification of the HAC1 Intron and Cloning and Isolation of the HAC1 Gene

##### *Y. lipolytica* HAC1 Splice Site.

On the basis of sequence homology between the intronic regions of HAC1 in *Yarrowia lipolytica* and the fungi *Trichoderma reesei* and *Aspergillus nidulans*, a potential splice site of the *Yarrowia lipolytica* HAC1 (Genbank: XM\_500811, Genolevures: Yali0B12716g) was identified. The 5' and 3' splice sites were predicted to be localized in a characteristic loop structure and the intron was calculated to be 29 bp long.

Primers were developed around the splice site in order to identify the intron. First strand cDNA was synthesized from the isolated mRNA from an UPR (unfolded protein response) induced (by means of growth in dithiothreitol (DTT)) and non-induced culture (negative control) with gene specific primers. PCR was then performed on first strand using prim-

ers HAC1FW06-003 and HAC1Rv06-001. Amplification products were analyzed on a 1.5% agarose gel.

A fragment of  $\pm 400$  bp was expected to be amplified for the non-induced cells; a 29 bp smaller fragment was expected to be amplified for the induced cells. Fragments of the correct size were obtained from the non-induced cells and the UPR induced cells. Two more amplification products were obtained for the UPR induced culture. The middle fragment was the same size as the band obtained for the non-induced culture and was interpreted as being unspliced HAC1. The lower, most prominent band was purified from the gel and cloned into a sequencing vector. After sequencing the construct, a sequence alignment was performed in order to identify the splice site (FIG. 15). From the sequence alignment it can be seen that the splice site is located at the position that was predicted from the comparison of the *Yarrowia lipolytica* and the fungal (*Trichoderma reesei* and *Aspergillus nidulans*) HAC1 sequences. The splice site is 29 bp long.

In order to isolate the active full length HAC1 sequence, primers were engineered to have restriction sites suitable for cloning into an expression vector. Primer sequences were as follows: Hac1yRv07-018: CCTAGGTCACCTCCAATCCCCAAACAGGTTGCTGACGCTCGACTCATAGTGAGCTAG ATCAGCAATAAAGTCG (SEQ ID NO:54) and HAC1Fw06-002:GGA TCC ATG TCT ATC AAG CGA GAA GAG TCC (SEQ ID NO:55). A 10 ml culture of yeast cells was incubated for 1.5 hours in the presence of 5 mM DTT to induce the UPR response. Following the incubation, RNA was isolated from the DTT-treated cells and first strand cDNA was prepared from the isolated RNA using reverse transcriptase and PCR using the cDNA as a template and the above primers. The PCR-amplified sequence containing the spliced HAC1 was inserted into the pCR-blunt-TOPO cloning vector using standard molecular biology techniques and sequenced.

##### *Pichia pastoris* HAC1 Splice Site.

On the basis of sequence homology of the intronic regions of the *Pichia pastoris* and *Saccharomyces cerevisiae* HAC1 genes, a potential splice site in the *Pichia pastoris* HAC1 gene was identified (FIG. 16). The 5' and 3' splice sites were predicted to be localized in a characteristic loop structure and the intron was calculated to be 322 bp in length.

Primers (HAC1Fw06-004 and HAC1Rv06-005) were developed around the predicted splice site in order to identify the intron (see Table 4). A fragment of 257 nucleotides was expected to be amplified when the intron is removed and a 579 bp fragment if intron is still present. First strand cDNA was synthesized from the isolated mRNA from an UPR induced and non-induced culture. The UPR was induced by adding 5 mM DTT to a 10 ml culture of exponentially growing cells. The cells were cultured in the presence of DTT for 1.5 hours. The amplification product was analyzed by 1.5% agarose gel electrophoresis. A fragment of approximately 257 bp was obtained from cDNA from both non-induced and induced cells.

TABLE 4

Primers		
Primer code	sequence 5'-->3'	Information
HAC1-Karl	GAATTCATGCCCGTAGATTC TTCTC (SEQ ID NO: 56) and EcoRI site	Forward primer Hac1 gene + start codon
HAC1Fw06-004	GAGTCTTCGGAGGATTC G (SEQ ID NO: 57)	Forward primer Hac1 gene region around 5' splice site

TABLE 4-continued

Primers		
Primer code	sequence 5'-->3'	Information
HAC1Rv06-005	CCTGGAAGAATACAAAGTC (SEQ ID NO: 58)	Reverse primer Hac1 gene near stop codon
HAC1Rv06-009	CCTAGGCTATTCTGGAAG AATACAAAGTC (SEQ ID NO: 59)	reverse primer Hac1 gene + stop codon and AviII site
ACTppFw07-007	GGTATTGCTGAGCGTATGC AAA (SEQ ID NO: 60)	Act1 forward primer for QPCR
ACTppRv07-003	CCACCGATCCATACGGAGT ACT (SEQ ID NO: 61)	Act1 reverse primer for QPCR
HAC1ppFw07-008	CGACCTGGAATCTGCACTT CAA (SEQ ID NO: 62)	Hac1 forward primer QPCR
HAC1ppRv07-004	CGGTACCACCTAAGGCTTC CAA (SEQ ID NO: 63)	Hac1 reverse primer QPCR
Kar2ppFw07-009	CCAGCCAAGTGTGTGATTC AA (SEQ ID NO: 64)	Kar2 forward primer QPCR
Kar2ppRv07-005	GGAGCTGGTGAATACCAG TCA (SEQ ID NO: 65)	Kar2 reverse primer QPCR

To verify the length of the unspliced *P. pastoris* HAC1 gene, PCR was performed on genomic DNA using primers HAC1-Karl and HAC1Rv06-005. The length of the obtained fragment was compared with the length of a PCR product obtained from the cDNA from an induced cell culture. The amplified fragment from the genomic DNA is about 300 bp longer than the amplicon derived from the cDNA using the same primers indicating that the intron is present in the genomic DNA sequence and absent from the spliced mRNA.

The cDNA fragment of 257 bp was isolated from the gel and cloned in a sequencing vector. The fragment was sequenced and an alignment was performed in order to identify the splice site (FIG. 17). To isolate and clone the spliced *P. pastoris* HAC1 gene, PCR primers were developed with restriction enzyme sites for cloning into an expression vector (HAC1-Karl and HAC1Rv06-009). A 10 ml culture was UPR-induced with 5 mM DTT for 1.5 hours. First strand cDNA was prepared from the isolated RNA using reverse transcriptase and PCR was subsequently performed on the cDNA template DNA using the above primers. The spliced HAC1 was isolated and cloned in pCR-blunt-TOPO cloning vector for sequencing. The spliced gene was also cloned under the control of the methanol inducible AOX1 promoter in the expression vector pBLHIS IX to obtain the vector pBLHIS IX ppHAC1 spliced. The correct insertion of the HAC1 gene into the expression vector was confirmed using PCR and restriction enzyme analysis.

In *Saccharomyces cerevisiae*, upon splicing, the coding sequence of the C-terminal 10 amino acids in the non-spliced mRNA is replaced with the coding sequence of 18 amino acids. In accordance, in *Pichia pastoris* it was revealed that the coding sequence of the C-terminal 45 amino acids in the non-spliced HAC1 are replaced upon splicing by the coding sequence of again 18 amino acids which are homologous to the ones from the *S. cerevisiae* sequence (FIG. 18).

#### Example 7

##### Transformation and Induction of Spliced HAC1 Gene into *Yarrowia lipolytica*

*Yarrowia lipolytica* cells (MTLY60 strain) were transformed with the vector "PYHMAXHAC1ylspliced" containing the spliced HAC1 cDNA (above) under the expression control of the hp4d promoter and the URA3 gene as a selection marker. Integration of the vector into the yeast genome was verified using PCR. The MTLY60 strain transformed with PYHMAXHAC1ylspliced was grown in a 2 ml culture in YPG at 28° C. for 24 hours. The cultured cells were washed twice with YNB, then diluted to OD<sub>600</sub> 0.6 and grown for 24 hours in YTG buffered with 50 mM phosphate buffer pH: 6.8. The cells were then diluted to OD<sub>600</sub> 0.2 and grown for 3 more generations in order to harvest the cells in the mid-exponential phase. To the pellet, 1 ml of RNApure™ solution was added to the cells along with 1 g of glass beads. Cells were broken by vigorous shaking RNA was extracted from the broken cells by adding 150 µl chloroform and precipitating the RNA with isopropanol. The extracted RNA was also treated with DNase to remove any coprecipitated DNA impurities.

First strand cDNA was prepared from 800 ng of the RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, Calif.) in a 20 µl total volume reaction. The equivalent of 20 ng RNA was used for real time PCR analysis to determine the amount of HAC1 mRNA in the cells. Real time PCR was run using SYBR® green as the detection reagent (fluorescent) (Eurogentec). In addition to designing primers for detecting the amount of HAC1 mRNA in the cells, primers were also designed to quantify the amount of ACT1 (household gene) and KAR2 (UPR responsive gene) genes as controls for the real time PCR. The relative amount of mRNA of each gene in the cells was calculated from the comparative threshold cycle values using Actin (a housekeeping gene) as the expression control. Induction of the UPR response by the cells was confirmed by measuring the expression of UPR. The

57

expression levels of KAR2 as well as HAC1 are higher in the strains expressing HAC1 under control of a constitutive promoter compared to the wild type strain MTLY60 (FIG. 39).

#### Example 8

##### Transformation and Induction of Spliced HAC1 Gene into *Pichia pastoris*

###### Media:

For the following experiments, three types of media were used: BMY (Buffered Medium for Yeast: 100 mM potassium phosphate pH:6.0/1.34% YNB without amino acids/1% Yeast extract/2% peptone); BGMY (Buffered Glycerol-complex Medium for Yeast: 100 mM potassium phosphate pH:6.0/1.34% YNB without amino acids/1% Yeast extract/2% peptone/1% glycerol); and BMMY (Buffered Methanol-complex Medium for Yeast: 100 mM potassium phosphate pH:6.0/1.34% YNB without amino acids/1% Yeast extract/2% peptone/0.5% glycerol).

*Pichia pastoris* cells were transformed according to the electroporation protocol from the *Pichia* Expression kit (Invitrogen Cat. No. K1710-01). The vector pBLHIS IX ppHAC1spliced was linearized in the HIS4 gene to target the construct to the HIS4 locus for integration. Ten micrograms of DNA was transformed into the yeast cells. The correct integration of the construct was validated using PCR on genomic DNA after isolation of single colonies (primers HAC1-Karl and HAC1Rv06-005). Fragments of 915 kb and 1237 kb were amplified from DNA obtained from the transformed cells, whereas in the non-transformants (cells without integration of the construct) a fragment of 1237 kb was amplified. Clones so identified as positive for integration of the plasmid were grown in 10 ml BMGY medium for 24 hours before induction. Cells were washed once with BMY. BMGY was added to non-induced cultures while BMY was added to the induced cultures. Every 12 hours, induced cultures were fed with 0.5% methanol (final concentration). Induction was performed for 24 hours after which cells were harvested by centrifugation. To prepare RNA, cells were combined with 1 ml RNAPure™ (Genhunter Corporation, Nashville, N.Y.) and 1 g of glass beads, and lysed by vigorous shaking RNA was extracted by the addition of 150 µl chloroform and precipitated with isopropanol. The extracted and precipitated RNA was DNase treated with RNase-free DNase obtained from Qiagen (Cat No. 79254). 400 ng of total RNA was subjected to reverse transcriptase reaction using an oligo dT primer and the Superscript II reverse transcriptase (Invitrogen, Cat. No. 18064-014). The equivalent of 20 ng RNA was used in a real-time PCR reaction. Primer sequences were designed by Primer Express software (Applied Biosystems) (see primer table for sequence). Real time PCR utilizing SYBR green fluorescent reagent (Eurogentec) was run in the iCycler machine from BioRad. The relative amounts of mRNA were calculated from the comparative threshold cycle values using the housekeeping gene actin as a control. Quantification of UPR is performed through expression analysis of the UPR-target gene KAR2. A 3 to 7 fold higher expression of KAR2 was obtained when comparing clones that were not induced as compared to the same clones that were induced with methanol (FIG. 19).

The relative amount of HAC1 mRNA from two additional clones 6 and clone 8 was determined by quantitative PCR and compared with the relative amount of mRNA of Kar2. A strong induction of HAC1 was observed in both clones. The relative amount of KAR2 mRNA appeared to correlate with

58

the relative amount of HAC1 mRNA, higher expression levels of HAC1 lead to higher expression level of KAR2 (FIG. 20).

Cell death studies of the methanol-induced cultures were performed using fluorescence flow cytometry (FFC) and compared to cell death of non-induced cultures. Ten thousand cells were measured per analysis. Cells were analyzed on the FACScalibur™ (Becton Dickinson) after 12, 36 and 48 hours of induction. No cell death was observed. The GlycoSwitchM5 (GSM5) strain has as main core type glycan structures mainly Man<sub>5</sub>GlcNAc<sub>2</sub> (structural formula IV; FIG. 4). In order to check if Hac1p induction has an influence on the N-glycan structure a DSA-FACE analysis was performed of 1 ml of the culture medium. The glycan profiles obtained after 48 hours of induction of spliced Hac1p are similar to the profile of the parental GSM5 strain.

A growth curve was made in order to check if the induction of Hac1p impairs the growth of *P. pastoris*. No growth defect was seen of the Hac1p induced strain compared to the empty vector transformed strain (FIG. 22).

#### Example 9

##### Expression of YIMNN6

In *S. cerevisiae*, MNN6 transfers phosphomannose residues to N-glycans. Therefore, overexpression of YIMNN6 in *Y. lipolytica* could lead to increased phosphorylation. Moreover, an additional effect on phosphorylation *Y. lipolytica* be obtained by over expressing YIMNN4 and YIMNN6. The YIMNN6 coding region (Genbank® Accession No. XM\_499811, Genolevures Ref: YAL10A06589g) was PCR amplified from the genome using PCR primers YIMNN6 BamHI fw (GCGGGATCCATGCACAACGTGCACGAAGC (SEQ ID NO:34)) and YIMNN6 AvrII rv (GCGCCTAGGCTACCAGTCACTATAGTTCTCC (SEQ ID NO:35)) and cloned in the pYHmAX expression vector for expression under control of the hp4d promoter (FIG. 21). The plasmid was transformed to the *Y. lipolytica* strain MTLY60 using zeta sequences to improve random integration. Secreted glycoproteins were collected from cell clones that grew on medium without uracil and the composition of the glycans synthesized the glycoproteins was analyzed using DSA-FACE. However, no increased phosphorylation was observed (FIG. 22).

#### Example 10

##### Effects of Hac1p Expression

Evaluation of Hac1p Overexpression on the Secretion of Heterologous Proteins.

Vectors containing the hygromycin resistance marker and the spliced HAC1 cDNA under control of the inducible AOX1 promoter (pPIChygppHAC1spliced) or under control of the constitutive GAP promoter (pGAPhygHAC1ppsplined) were transformed to a GS115 strain expressing a mL-10 protein under the control of the inducible AOX1 promoter. *P. pastoris* cells were transformed according to the electroporation protocol from the *Pichia* Expression kit (Invitrogen Cat. No. K1710-01, Invitrogen, Carlsbad, Calif.). The vectors were linearized in the AOX1 or GAP promoter to target the integration of the Hac1p gene to respectively the AOX1 or GAP locus. Integration of the plasmid into the host genome was confirmed using PCR.

Precultures (5 ml) from positive identified clones were grown in YPD for 24 hours. The concentration (OD) at a wavelength of 600 nm (OD<sub>600</sub>) of the cells in the cultures was

measured and cultures were diluted to an OD<sub>600</sub> of 1 in 2 ml of BMGY media in each well of a 24 well plate. Cultures were grown in BMGY for 48 hours, washed twice with BMY, and then induced for 24 hours in BMMY. Every 8 to 12 hours, cultures were re-fed with medium containing 1% methanol (final concentration). After induction, the supernatant of the cells was harvested and the protein from 1 ml of the supernatant was precipitated using trichloroacetic acid (TCA). The precipitated protein was subjected to 15% SDS-PAGE.

From the SDS-PAGE, clonal variation in the expression of at least one protein—mIL-10—was observed between the different clones. For example, for the clones expressing the Hac1p protein constitutively (under control of GAP promoter), no improvement in expression level was observed, whereas for the clones expressing the Hac1p inducibly (AOX1 promoter), two clones could be identified that exhibited higher expression levels of the mIL-10 protein (FIG. 40 and FIG. 41). Expression of mIL-10 by each of the clones was compared to the expression of mIL-10 produced by a reference GS115 mIL-10 expressing strain.

A new induction was performed for these clones. A pre-culture grown for 24 hours was diluted to OD 1 in 20 ml BMGY in a baffled flask. Cells were grown for 48 hours in BMGY, washed twice, and then induced in BMMY. Cultures were re-fed with medium containing 1% methanol every 8-12 hours. After induction, the supernatant of the cells was harvested and the protein from 1 ml of the supernatant was precipitated using TCA. Prior to subjecting the precipitated protein to 15% SDS-PAGE, the protein was treated with PNGase F (or not) to remove all glycosylation (FIG. 41). SDS-PAGE resolved proteins from the supernatant of Hac1p-expressing strains contained a prominent band of 75 kDa, which is not present in the reference strain. This band was identified by means of mass spectrometry as being Kar2p, which is the most prominent UPR target gene. It could be shown using the cytokine bead array (CBA) that simultaneous inducible expression of the Hac1p and the mIL-10 protein can lead to a 2 fold higher expression of the mIL-10 protein (clone 1, FIG. 41). CBA was performed on endoH treated mIL-10 protein.

Evaluation of Hac1p Overexpression on the Surface Expression of Heterologous Proteins.

Vectors containing the hygromycin resistance marker and the spliced HAC1 cDNA under control of the inducible AOX1 promoter (pPlChyppHAC1spliced) or under control of the constitutive GAP promoter (pGAPHygHAC1ppspliced) were transformed to GlycoswitchMan5 strains expressing a mature human interferon-beta/alpha-agglutinin fusion protein, a mature mouse interferon gamma/alpha-agglutinin fusion protein, a mature human erythropoietin/alpha-agglutinin fusion protein, or a fusion protein of alpha-agglutinin and the lectin-like domain of mouse thrombomodulin, each of which were under the control of the inducible AOX1 promoter. *P. pastoris* cells were transformed according to the electroporation protocol from the *Pichia* Expression kit (Invitrogen Cat. No. K1710-01). The vectors were linearized in the AOX1 or GAP promoter to target the Hac1p gene to respectively the AOX1 or GAP locus for integration. Integration of the plasmid into the host genome was confirmed using PCR.

Precultures (5 ml) from positive identified clones were grown in YPD for 24 hours. The OD<sub>600</sub> was measured and cultures were diluted to OD<sub>600</sub> of 1 in 2 ml BMGY in each well of a 24 well plate. The cultures were grown in BMGY for 24 hours, washed twice with dionized water, and then induced (using culture medium containing 1% methanol) for 24 hours in BMMY. Surface expression was demonstrated by indirect immunostaining with an antibody specific for the V5-epitope,

which is fused C-terminally to the V<sub>H</sub>H coding sequence. After induction, 10<sup>7</sup> cells in 1 ml PBS (pH 7.2), supplemented with 0.1% bovine serum albumin (PBS/BSA), were incubated with 1 µl/ml of the anti-V5 antibody (1 µg/µl; Invitrogen), washed with PBS/BSA, and incubated with 1 µl/ml Alexa fluor 488-labeled goat anti-mouse IgG (1 µg/µl; Molecular Probes). After washing twice with PBS/BSA, the cells were analyzed by flow cytometry (Table 5).

TABLE 5

MFI values determined by flow cytometry			
Expressed Protein	Wild-type <i>Pichia</i>	<i>Pichia</i> + AOX GAP	<i>Pichia</i> + GAP HAC
mouse Interferon-gamma	36.6	19.9	42.8
human EPO	59.5	45.8	66.5
interferon-beta	22.6	12.4	14.4
human thrombomodulin	95.5	184.1	67.8

MFI = Mean Fluorescence Intensity obtained from the flow cytometry analysis.

For the strains expressing the Hac1p protein constitutively no improvement, or very minor differences, could be observed in surface expression levels for all four proteins compared to reference strains expressing the surface protein alone. In cells expressing human interferon-beta, a significant reduction of surface expression levels was observed. For the strains overexpressing the inducible Hac1p (Table 5) the following could be observed: 1) in the human interferon-gamma surface expressing strain, a 1.8-fold lowering of the surface expression levels could be observed compared to the reference strain expressing alone the human interferon-beta a-agglutinin fusion; 2) for the strain surface-expressing human erythropoietin a-agglutinin fusion protein, a 1.3-fold lowering of the surface expression levels could be observed compared to the reference strain; 3) in the strain surface expressing human interferon-beta, no difference of the surface expression levels could be observed compared to the reference strain; and 4) in the strain surface expressing mouse thrombomodulin lectin-like domain, an 1.9-fold increase of the surface expression levels could be observed compared to the reference strain.

Effect of Overexpression of Hac1p on Phospholipid Synthesis.

To determine whether overexpression of the Hac1p product (produced from the spliced HAC1 cDNA) had an effect of lipid metabolism in *P. pastoris*, cells were transformed with the above-described spliced HAC1 cDNA and the effect of Hac1p on lipid metabolism in the cells was determined by electron microscopy analysis. Cells were grown for 48 hours on BMGY, washed once with PBS, and then grown for another 48 hours on BMMY. The cells were next cultured in medium containing 1% methanol every 8 to 12 hours. The cells were then prepared for electron microscopy according to the method of Baharaeen (Baharaeen et al. (2004) Mycopathologia). Briefly, a primary fixative containing glutaraldehyde (3%) and para-formaldehyde (1.5% buffered in 0.05 M sodium cacodylate at pH 7.2) was contacted with the cells for 2 hours on ice. The cells were then washed three times for 20 minutes with 0.05 M sodium cacodylate. After washing, the cells were contacted with a 6% potassium permanganate solution for one hour at room temperature and then washed with 0.05 M sodium cacodylate three times for 20 minutes. The results of the experiment are presented in FIG. 54. Overexpression of the Hac1p product (produced from the spliced HAC1 cDNA) in *P. pastoris* lead to the formation of discrete

regions of stacked membranes as can be shown in the electron micrograph (EM) depicted in FIG. 54. These results demonstrate that overexpression of Hac1p, by way of its transcriptional activation of genes involved in lipid metabolism, indeed has a strong effect on lipid metabolism in *P. pastoris*.

#### Example 11

##### Expression of ManHDEL

For Man<sub>5</sub>GlcNAc<sub>2</sub> to be bound to glycoproteins expressed by the Δoch1 strain, an α-1,2-mannosidase can be expressed to cleave Man<sub>8</sub>GlcNAc<sub>2</sub> to Man<sub>5</sub>GlcNAc<sub>2</sub> (i.e., Golgi type α-1,2-mannosidase activity). This mannosidase should be targeted to the secretion system. *Trichoderma reesei* α-1,2-mannosidase (Genbank® accession no. AF212153), fused to the *S. cerevisiae* prepro mating factor and tagged with a HDEL sequence, is able to trim Man<sub>8</sub>GlcNAc<sub>2</sub> to Man<sub>5</sub>GlcNAc<sub>2</sub> in vivo in *Pichia pastoris* as well as in *Trichoderma reesei* and *Aspergillus niger*. An expression construct was made to overexpress MfManHDEL (*S. cerevisiae* α-mating factor prepro fused to *Trichoderma reesei* α-1,2-mannosidase tagged with an HDEL sequence) in *Y. lipolytica* under control of the constitutive hp4d promoter (FIG. 23). The expression cassette was transformed into the cells after digestion of the plasmid pYHmAXManHDEL with the restriction enzyme NotI, followed by isolation of the desired fragment using agarose-gel electrophoresis.

Glycans derived from mannoproteins from the transformed cells were analysed using DSA-FACE. Only a minor fraction of Man<sub>8</sub>GlcNAc<sub>2</sub> was converted to Man<sub>5</sub>GlcNAc<sub>2</sub> (FIG. 24). Incomplete conversion of Man<sub>8</sub>GlcNAc<sub>2</sub> to Man<sub>5</sub>GlcNAc<sub>2</sub> could have been due to a non-optimal secretion signal. Therefore, the *Saccharomyces cerevisiae* secretion signal was replaced with the secretion signal derived from the well expressed *Yarrowia lipolytica* LIP2 (LIP2pre). The LIP2pre sequence was made by hybridizing the synthetic oligonucleotides LIP2pre fw GATCCATGAAGCTTTCACCATCCTCTTCACAGCCTGCGCTACCCTGGCCGCGGTAC (SEQ ID NO:66) and Lip2prepro rv GTACCGGCGGCCGCTTCTGGAGAACTGCGGCTCAGAAGGAGTGATGGGGGAAGG GAGGGCGGC (SEQ ID NO:67) and cloning the DNA into pYLHmA vector (at the BamHI/AvrII sites) resulting in the following construct: pYLHdL2pre. The ManHDEL coding sequence was PCR amplified from pGAPZMfManHDEL using oligonucleotides ManHDEL Eco47III fw (GGCAGCGCTACAAAACGTGGATCTCCCAAC (SEQ ID NO:68)) and ManHDEL AvrII rv (GGCCCTAGGTTACAACTCGTCGTGAGCAAG (SEQ ID NO:69)) and cloned in pYLHdL2pre. The construction strategy is depicted in FIGS. 25A and 25B. The expression cassette (with L2preManHDEL under control of the constitutive promoter hp4d) was transformed to *Yarrowia lipolytica* Δoch1 strain after digestion of the plasmid with NotI and isolation of the correct fragment (see above). Glycans derived from secreted proteins were analysed via DSA FACE. Some conversion of Man<sub>8</sub>GlcNAc<sub>2</sub> to Man<sub>5</sub>GlcNAc<sub>2</sub> occurred, but the reaction was incomplete (Man<sub>8</sub>GlcNAc<sub>2</sub> was present as well as intermediate products Man<sub>7</sub>GlcNAc<sub>2</sub> and Man<sub>6</sub>GlcNAc<sub>2</sub>; FIG. 26).

To further improve the trimming of Man<sub>8</sub>GlcNAc<sub>2</sub>, Man<sub>7</sub>GlcNAc<sub>2</sub>, and Man<sub>6</sub>GlcNAc<sub>2</sub> to Man<sub>5</sub>GlcNAc<sub>2</sub>, the *Trichoderma reesei* α-1,2 mannosidase was codon optimized for expression in *Yarrowia lipolytica* (SEQ ID NO:9; FIG. 42) and fused to the LIP2 pre signal sequence. This fusion construct was expressed under control of 4 different promoters: (i) hp4d, (ii) GAP (SEQ ID NO:10; FIG. 43), (iii) POX2, and

(iv) TEF1. Final expression plasmids were named pYLHUXdL2preManHDEL (SEQ ID NO: 11; FIGS. 44A-C) pYLGUXdL2preManHDEL (SEQ ID NO:12; FIGS. 45A-C) pYLPUXdL2preManHDEL (SEQ ID NO:13; FIGS. 46A-C) pYLTUXdL2preManHDEL (SEQ ID NO:14; FIGS. 47A-C). All 4 plasmids were transformed to *Yarrowia lipolytica* MTLY60 Δoch1 strain (described in example 2) after cutting the plasmid with NotI and isolation of the fragment containing the ManHDEL expression cassette. Transformed strains with the ManHDEL under control of the hp4d, GAP and TEF promoter (plasmids pYLHUXdL2preManHDEL, pYLGUXdL2preManHDEL and pYLTUXdL2preManHDEL) were grown in YPD.

Glycans derived from secreted proteins of transformed strains were analyzed by DSA FACE. Results are represented in FIG. 48. Alternatively, transformants (including a transformant that had integrated the pYLPUXdL2preManHDEL plasmid) were grown in medium containing oleic acid (protein production conditions) and glycans were analysed via DSA-FACE. Data for one of the vectors, pYLTUXdL2preManHDEL, are presented in FIG. 49. As can be concluded from the data, by 48 hours of culture, almost all glycans are converted to Man<sub>5</sub>GlcNAc<sub>2</sub>.

#### Example 12

##### Culturing Conditions for POX2 Promoter Controlled Gene Expression

Cultures were started from a single colony of a fresh plate and grown overnight in 10 mL YPD at 28° C. in a 50 mL tube in an orbital shaker at 250 rpm. Next, a 250 mL shake flask containing 22 mL of production medium (including 2.5 mL oleic acid emulsion) was inoculated with the preculture at a final OD<sub>600</sub> of 0.2. This culture was incubated at 28° C. in an orbital shaker at 250 rpm. Samples of the culture were taken at various time points over a 96 hour culture. The oleic acid emulsion (20%) was made the method as follows:

Add to a sterile 50 ml vessel;  
20 ml sterile water;  
5 ml oleic acid; and  
125 μl Tween 40.

Sonication resulting in the formation of the emulsion was performed for one minute at 75 Hz.

The production medium consisted of the following:

1% yeast extract;  
2% trypton;  
1% glucose; and  
50 mM phosphate pH 6.8.

#### Example 13

##### Expression of Human Glucocerebrosidase

Human glucocerebrosidase (GLCM, Swiss Prot entry nr: P04062) was chemically synthesized as a codon-optimized cDNA for expression in *Yarrowia lipolytica* (SEQ ID NO:15; FIG. 50).

The coding sequence for the mature protein was fused to the coding sequence of the LIP2 pre signal sequence. This fusion construct was cloned under control of the oleic acid inducible POX2 promoter. The resulting plasmid was named pYLPUXL2preGLCM (=pRAN21)). Before transformation, the plasmid was digested with NotI and the fragment containing the expression cassette was isolated and transformed to *Yarrowia lipolytica* strain MTLY60, MTLY60Δoch1 (de-

## 63

scribed in Example 2 above), and MTLY60 $\Delta$ och1ManHDEL (described in Example 11). Transformants obtained in these three strains were grown as described in Example 12. Proteins were precipitated from the supernatant as described above, subjected to SDS-PAGE, and immunoblotted using a rat monoclonal anti-glucocerebrosidase antibody (Alessandrini et al. (2004) J. Invest. Dermatol 23(6):1030-6). An exemplary immunoblot analysis is depicted in FIG. 51. It can be appreciated from FIG. 51 that in a och1 disrupted strain no smearing occurs (lanes 1, 2, and 3), whereas heterogeneity of the protein is seen as a smear in WT cells (lanes 4 and 6). No smearing of protein was observed in protein obtained from a strain of yeast expressing ManHDEL. These results demonstrate that a more homogeneous population of a target protein can be obtained using the genetically engineered *Yarrowia lipolytica* cells MTLY60 $\Delta$ och1 and MTLY60 $\Delta$ och1ManHDEL.

## Example 14

## Expression of Human Erythropoietin

Human erythropoietin (Epo, Swiss Prot entry nr: P01588) encoding cDNA was chemically synthesized codon optimized for expression in *Yarrowia lipolytica* (SEQ ID NO:16; FIG. 52). The cDNA coding sequence for the mature protein was fused to the coding sequence of the LIP2 pre signal sequence. This fusion construct was cloned under control of the oleic acid inducible POX2 promoter. The resulting plasmid was named pYLPUXL2prehuEPO. Before transformation the plasmid was cut NotI and the fragment containing the expression cassette was isolated and transformed to *Yarrowia lipolytica* strain MTLY60 $\Delta$ och1 (described in Example 2). Transformant candidates were grown as described in Example 12 and secreted proteins were analysed by western blot after SDS PAGE using a monoclonal mouse anti human Epo antibody obtained from R&D systems (clone AE7A5). The EPO product obtained from the cells exhibited very homogenous glycosylation.

## Example 15

Expression of Human  $\alpha$ -Galactosidase A

Human  $\alpha$ -galactosidase A (AGAL, Swiss Prot entry nr: P06280) encoding cDNA was chemically synthesized as a

## 64

codon-optimized cDNA for expression in *Yarrowia lipolytica* (SEQ ID NO:17; FIG. 53). FIGS. 12A-12H contain the cloning strategy of the galactosidase.

The cDNA coding sequence for the mature protein was fused to the coding sequence of the LIP2 pre signal sequence. This fusion construct was cloned under control of the oleic acid inducible POX2 promoter. The resulting plasmid was named pYLPUXL2preGalase). Before transformation the plasmid was cut NotI and the fragment containing the expression cassette was isolated and transformed to *Yarrowia lipolytica* strain MTLY60 and MTLY60 $\Delta$ och1MNN4 (described in Example 4). Transformants obtained in these two strains were grown as described in Example 12. Extracellular proteins obtained from transformants were analyzed by immunoblot after SDS-PAGE analysis. Two antibodies specific for  $\alpha$ -galactosidase A (a chicken polyclonal antibody obtained from Abcam (ab28962) and a rabbit polyclonal antibody obtained from Santa Cruz Biotechnology (sc-25823)) were used to detect the expressed human  $\alpha$ -galactosidase A protein.

## Example 16

Expression of Mannosidase in WT *Yarrowia lipolytica*

To determine whether expression of MannosidaseHDEL alone (that is in cells containing a functional OCH1 gene) could lead to a more homogenous glycosylation of proteins expressed by fungal cells, an expression cassette containing a nucleic acid encoding MannosidaseHDEL (see Example 11) was transformed into wild-type *Yarrowia lipolytica* pold cells. Glycans derived from secreted proteins obtained from the cells were analysed by DSA-FACE (FIG. 55). The analyzed glycans consisted mainly of Man<sub>5</sub>GlcNAc<sub>2</sub> and a minor part Man<sub>6</sub>GlcNAc<sub>2</sub>. These results demonstrate that expression of MannosidaseHDEL alone, in the absence of any disruption of the OCH1 gene, leads to a more homogenous glycosylation of proteins expressed by *Yarrowia lipolytica*.

## OTHER EMBODIMENTS

While the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

## SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 81

<210> SEQ ID NO 1

<211> LENGTH: 358

<212> TYPE: DNA

<213> ORGANISM: *Yarrowia lipolytica*

<400> SEQUENCE: 1

```

gggtctcgcca ggcgcgccac cctcttcaac cagcataatg agaccgtcgt ccccgactct    60
cctattgtga agaccgagga agtcgactct acaaactttc tctccacac ggagtcctcc    120
tcccccccg aactagctga gagcactggc tcaggctcgc catcgtcgac tctgtcctgc    180
gacgaaactg attatcttgt ggaccgggcg cgtcacccag cagcgtcgcg acaagatttc    240
wttttcatca aggacgagcc cggttgacgac gagcttgga c tgcattggact gtcggatgac    300
ttcacctgt ttgaagacaa caagcagcct gccacgacac actttattgc tgatctag    358

```

-continued

<210> SEQ ID NO 2  
 <211> LENGTH: 915  
 <212> TYPE: DNA  
 <213> ORGANISM: *Pichia pastoris*

<400> SEQUENCE: 2

```

atgcccgtag attcttctca taagacagct agcccacttc cacctcgtaa aagagcaaag      60
acggaagaag aaaaggagca gcgtcgagtg gaacgtatcc tacgtaatag gagagcggcc      120
catgcttcca gagagaagaa acgaagacac gttgaatttc tggaaaacca cgtcgtcgac      180
ctggaatctg cacttcaaga atcagccaaa gccactaaca agttgaaaga aatacaagat      240
atcattgttt caaggttgga agccttaggt ggtaccgtct cagatttgga tttaacagtt      300
ccggaagtgc attttcccaa atcttctgat ttggaaccca tgtctgatct ctcaacttct      360
tcgaaatcgg agaaagcatc tacatccact cgcagatctt tgactgagga tctggacgaa      420
gatgacgtcg ctgaatatga cgacgaagaa gaggacgaag agttaccag gaaaatgaaa      480
gtcttaaacg acaaaaacaa gagcacatct atcaagcagg agaagttgaa tgaacttcca      540
tctcctttgt catccgattt ttcagacgta gatgaagaaa agtcaactct cacacattta      600
aagttgcaac agcaacaaca acaaccagta gacaattatg tttctactcc tttgagtctt      660
ccggaggatt cagttgattt tattaacca ggtaacttaa aaatagagtc cgatgagaac      720
ttcttggtga gttcaaatac ttacaaata aaacacgaaa atgacaccga ctacattact      780
acagctccat caggttccat caatgatttt ttaattctt atgacattag cgagtcgaat      840
cggttgcatc atccagcagc accatttacc gctaatgcat ttgatttaaa tgactttgta      900
ttcttcagg aatag                                     915

```

<210> SEQ ID NO 3  
 <211> LENGTH: 305  
 <212> TYPE: PRT  
 <213> ORGANISM: *Yarrowia lipolytica*

<400> SEQUENCE: 3

```

Met Ser Ile Lys Arg Glu Glu Ser Phe Thr Pro Thr Pro Glu Asp Leu
  1           5           10          15
Gly Ser Pro Leu Thr Ala Asp Ser Pro Gly Ser Pro Glu Ser Gly Asp
          20          25          30
Lys Arg Lys Lys Asp Leu Thr Leu Pro Leu Pro Ala Gly Ala Leu Pro
          35          40          45
Pro Arg Lys Arg Ala Lys Thr Glu Asn Glu Lys Glu Gln Arg Arg Ile
          50          55          60
Glu Arg Ile Met Arg Asn Arg Gln Ala Ala His Ala Ser Arg Glu Lys
          65          70          75          80
Lys Arg Arg His Leu Glu Asp Leu Glu Lys Lys Cys Ser Glu Leu Ser
          85          90          95
Ser Glu Asn Asn Asp Leu His His Gln Val Thr Glu Ser Lys Lys Thr
          100         105         110
Asn Met His Leu Met Glu Gln His Tyr Ser Leu Val Ala Lys Leu Gln
          115         120         125
Gln Leu Ser Ser Leu Val Asn Met Ala Lys Ser Ser Gly Ala Leu Ala
          130         135         140
Gly Val Asp Val Pro Asp Met Ser Asp Val Ser Met Ala Pro Lys Leu
          145         150         155         160

```

[illegible]

```
<210> SEQ ID NO 4
<211> LENGTH: 304
<212> TYPE: PRT
<213> ORGANISM: Pichia pastoris
```

<400> SEQUENCE: 4

Met 1	Pro	Val	Asp	Ser 5	Ser	His	Lys	Thr	Ala 10	Ser	Pro	Leu	Pro	Pro	Arg
Lys	Arg	Ala	Lys 20	Thr	Glu	Glu	Glu	Lys 25	Glu	Gln	Arg	Arg	Val 30	Glu	Arg
Ile	Leu	Arg 35	Asn	Arg	Arg	Ala	Ala 40	His	Ala	Ser	Arg	Glu 45	Lys	Lys	Arg
Arg	His 50	Val	Glu	Phe	Leu	Glu 55	Asn	His	Val	Val	Asp 60	Leu	Glu	Ser	Ala
Leu 65	Gln	Glu	Ser	Ala	Lys 70	Ala	Thr	Asn	Lys	Leu 75	Lys	Glu	Ile	Gln	Asp 80
Ile	Ile	Val	Ser 85	Arg	Leu	Glu	Ala	Leu 90	Gly	Gly	Thr	Val	Ser	Asp 95	Leu
Asp	Leu	Thr	Val 100	Pro	Glu	Val	Asp	Phe 105	Pro	Lys	Ser	Ser	Asp 110	Leu	Glu
Pro	Met	Ser	Asp 115	Leu	Ser	Thr	Ser 120	Ser	Lys	Ser	Glu	Lys 125	Ala	Ser	Thr
Ser	Thr 130	Arg	Arg	Ser	Leu	Thr 135	Glu	Asp	Leu	Asp 140	Glu	Asp	Asp	Val	Ala
Glu 145	Tyr	Asp	Asp	Glu	Glu 150	Glu	Asp	Glu	Glu	Leu 155	Pro	Arg	Lys	Met	Lys 160
Val	Leu	Asn	Asp 165	Lys	Asn	Lys	Ser	Thr	Ser 170	Ile	Lys	Gln	Glu	Lys 175	Leu
Asn	Glu	Leu 180	Pro	Ser	Pro	Leu	Ser 185	Ser	Asp	Phe	Ser	Asp 190	Val	Asp	Glu
Glu	Lys 195	Ser	Thr	Leu	Thr	His	Leu 200	Lys	Leu	Gln	Gln	Gln 205	Gln	Gln	Gln
Pro 210	Val	Asp	Asn	Tyr	Val	Ser 215	Thr	Pro	Leu	Ser 220	Leu	Pro	Glu	Asp	Ser

-continued

---

Val Asp Phe Ile Asn Pro Gly Asn Leu Lys Ile Glu Ser Asp Glu Asn  
 225 230 235 240

Phe Leu Leu Ser Ser Asn Thr Leu Gln Ile Lys His Glu Asn Asp Thr  
 245 250 255

Asp Tyr Ile Thr Thr Ala Pro Ser Gly Ser Ile Asn Asp Phe Phe Asn  
 260 265 270

Ser Tyr Asp Ile Ser Glu Ser Asn Arg Leu His His Pro Ala Ala Pro  
 275 280 285

Phe Thr Ala Asn Ala Phe Asp Leu Asn Asp Phe Val Phe Phe Gln Glu  
 290 295 300

<210> SEQ ID NO 5  
 <211> LENGTH: 387  
 <212> TYPE: DNA  
 <213> ORGANISM: Yarrowia lipolytica

<400> SEQUENCE: 5

```

ggctctcgcca gcgcgcccac cctcttcaac caccgataatg agaccgtcgt ccccgactct    60
cctattgtga agaccgagga agtcgactct acaaactttc tcctccacac ggagtctctc    120
tcccccccg aactagctga gagcactggc tcaggctcgc catcgctgac tctgtcctgc    180
gacgaaactg attatcttgt ggaccgggcg cgtcatccag cagtgatgac tgtcgcaact    240
actgaccagc agcgtcggca caagatttca ttttcatcaa ggacgagccc gttgacgacg    300
agcttggaact gcattgactg tcggatgact tcacctggtt tgaagacaac aagcagcctg    360
cccagcacga ctttattgct gatctag                                     387
  
```

<210> SEQ ID NO 6  
 <211> LENGTH: 1237  
 <212> TYPE: DNA  
 <213> ORGANISM: Pichia pastoris

<400> SEQUENCE: 6

```

atgccgtag attcttctca taagacagct agcccacttc cacctcgtaa aagagcaaag    60
acggaagaag aaaaggagca gcgtcgagtg gaacgtatcc tacgtaatag gagagcggcc    120
catgcttcca gagagaagaa acgaagacac gttgaatttc tggaaaacca cgtcgtcgac    180
ctggaatctg cacttcaaga atcagccaaa gccactaaca agttgaaaga aatacaagat    240
atcattgttt caaggttgga agccttaggt ggtaccgtct cagatttgga tttacagtt    300
ccggaagtgc attttcccaa atcttctgat ttggaaccca tgtctgatct ctcaacttct    360
tcgaaatcgg agaaagcatc tacatccact cgcagatctt tgactgagga tctggacgaa    420
gatgacgtcg ctgaatatga cgacgaagaa gaggacgaag agttaccag gaaaatgaaa    480
gtctttaaag acaaaaaaaa gagcacatct atcaagcagg agaagttgaa tgaacttcca    540
tctcttttgt catccgattt ttcagacgta gatgaagaaa agtcaactct cacacattta    600
aagttgcaac agcaacaaca acaaccagta gacaattatg tttctactcc tttgagtctt    660
ccggaggatt cagttgattt tattaacca ggtaacttaa aaatagagtc cgatgagaac    720
ttcttggtga gttcaaatat tttaaaaata aaacacgaaa atgacaccga ctacattact    780
acagctccat caggttccat caatgatttt tttaattctt atgacattag cgagtcgaat    840
cggttgcatc atccagcagt gatgacggat tcattctttac acattacagc aggtccatc    900
ggctttttct ctttgatttg ggggggggaa agttctgtag cagggaggcg cagttcagtt    960
ggcacatatc agttgacatg catagcgatc aggtgattgc tcttgtaaac tggtatgcca   1020
  
```

-continued

---

```

agtcaaccgt ttctggcgta cctactactc tactttaact cttgaattga gatgaaacaa 1080
tcgcccttgt tcgagacgcg tagttaattt aagatgaatc aatttgcaac aaaagaacaa 1140
acgaaaaaga taacagcaaa cttaagtttc gtggtctgca gcaccattta ccgctaattgc 1200
atctgattta aatgactttg tattcttcca ggaatag 1237

```

```

<210> SEQ ID NO 7
<211> LENGTH: 2810
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetically generated oligonucleotide

```

```

<400> SEQUENCE: 7

```

```

gtaaagcacg agaacttcaa gacctgttct cagtctggct tctgtaagcg aaaccgagcc 60
ttcgccgacg acgccgctgc ccagggtctt tcttgggcct ctccctacga gctggactct 120
tcttctatcc agttcaagga cggccagctg caccgaccca tcctgaagtc tgtgtctccc 180
aacgagaagg tgaagctgcc cctgggtgtg tctttcctgg agtctggcgc cgtctgagtg 240
gtggtggacg aggagaagcg aatgaacggc gacatccagc tgcgacacga ctctaaggcc 300
cgaaaggagc gatacaacga ggccgagaag tgggtgctgg tcggcggcct ggagctgtct 360
aagaccgcca cctgcgacc cgagaccgag tctggttcca cccgagtgtc gtacggcccc 420
gacaaccagt tcgaggccgt gatccgacac gcccccttct ctgccgactt caagcgagat 480
ggccagaccc acgtgcagct gaacaacaag ggctacctga acatggagca ctggcgaccc 540
aaggtggagg tggagggcga gggtagagcg cagacccagg aggacgagtc tacctggtag 600
gacgagtctt tcggcggcaa caccgacacc aagccccgag gccccgagtc tgtgggcttg 660
gacatcacct tccccggcta caagcacgtg ttcggcatcc ccgagcacgc cgactccctg 720
tctctgaagg agacccgagg cggcgagggc aaccacgagg agccctaccg aatgtacaac 780
gccgacgtgt tcgagtaaga gctgtcctct cccatgaccc tgaacggcgc catccccttc 840
atgcaggccc accgaaagga ctctaccgtg ggcgtgttct ggctgaacgc cgcgagagcc 900
tgggtggaca tcgtgaagtc tacctcttct cccaaccccc tggccctggg cgtgggagcc 960
accaccgaca cccagtctca ctggttctct gagtctggcc agctggagct gttcgtgttc 1020
ctgggccccca cccccagga gatttctaag acctacggcg agctgaccgg ctacaccag 1080
ctgccccagc acttcgccat tgcctaccac cagtgtcgat ggaactacat caccgacgag 1140
gacgtgaagg aggtggaccg aaacttcgac aagtaccaga tcccctacga cgtgatctgg 1200
ctggacatcg agtacaccga cgaccgaaag tacttcacct gggacccccct gtctttcccc 1260
gaccccatct ctatggagga gcagctggac gagtctgagc gaaagctggc cgtgatcatc 1320
gacccccaca tcaagaacca ggacaagtac tctatcgtgc aggagatgaa gtctaaggac 1380
ctggccacca agaacaagga cggcgagatt tacgacggct ggtgttggcc cggctcttct 1440
cactggatcg acaccttcaa ccccgctgcc atcaagtggc ggggtgtctct gttcaagttc 1500
gacaagttca agggcacccct gtctaactgt ttcactctga acgacatgaa cgagccctct 1560
gtgttcaacg gccccgagac caccatgccc aaggacaacc tgcaccacgg caactgggag 1620
caccgagaca tccacaacgt gcacggcatc accctgggtg acgcccaccta cgacgccttg 1680
ctggagcgaa agaagggcga gatccgacga cccctcatcc tgacccgata ttactacgcc 1740
ggtgcccagc gaatgtctgc catgtggacc ggcgacaacc aggccacctg ggagcacctg 1800

```

-continued

---

gccgcctcta tccccatggt gctgaacaac ggaatcgccg gcttcccctt cgccggtgcc	1860
gacgtgggag gcttcttcca gaaccctctt aaggagctgc tgaccgatg gtatcaggcc	1920
ggcatctggt atcctttctt ccgagccac gccacatcg acaccgacg acgagagccc	1980
tacctgatcg ccgagcccca ccgatctatc atctctcagg ccatecgact gcgataccag	2040
ctgctgcccc cctggtagac cgccctccac gaggcctctg tgaacggcat gccatcgctg	2100
cgacccagc actacgcccc ccctgggac gaggcgggt tcgcatcga cgaccagctg	2160
tacctgggct ctaccggcct gctggccaag cccgtgggtg ctgaggaggc caccaccgcc	2220
gacatctacc tgggtgacga cgagaagtac tacgactact tcgactacac cgtgtaccag	2280
ggcgctggca agcgacacac cgtgccgct cccatggaga ccgtgccctt gctgatgcag	2340
ggcggccacg tgatcccccg aaaggaccga ccccgacgat cttctgccct gatgcgatgg	2400
gacccctaca ccctgggtgt ggtgctggac aagaacggcc aggcggacgg ctctctgtac	2460
gtggacgacg gcgagacctt cgactacgag cgaggcgctt acatccatcg acgattccga	2520
ttccaggagt ctgccctggt ctccgaggac gtgggcacca agggcccca gaccgcccag	2580
tacctcaaga ccatggccaa cgtgcgagtg gagcgagtgg tggctgtgga ccccccaa	2640
gagtggcagg gcaagacctc tgtgaccgtg atcgaggacg gcgcctctgc cgctctacc	2700
gcctccatgc agtaccactc tcagcccgac ggcaaggccg cctacgccgt ggtgaagaac	2760
cccaacgtgg gcatcgccaa gacctggcga atcgagttct aatagcctag	2810

&lt;210&gt; SEQ ID NO 8

&lt;211&gt; LENGTH: 1651

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Synthetically generated oligonucleotide

&lt;400&gt; SEQUENCE: 8

agcgctggcg acgcctcttc tcgacccga ggcgtgggcc ccgagttcgc caagttctac	60
aaggacacca ccaccttcac ctgtatctct caccocgcca tccagattcc cttctctgcc	120
gtgaacgacg actactgtga ctgtcccgac ggctctgacg agcccgccac ctctgcctgt	180
gcctttctgt ctcgaaactc tgccctgacc cccggtgagc gacccgatc tgacgacctg	240
gagctgacct ctgccctgcc cggcttctac tgtaagaaca agggccacaa gcccggtac	300
gtgcccttcc agcgagttaa cgatggcatc tgtgactacg agctgtgttg tgacggatcg	360
gatgagtggt ctgaccccg cgaaccaag tgtgaggaca agtgtaagga gatcggaag	420
gagtggcgaa agaaggagga gaagcgacag aagtctatga ccgccgctct gaagaagaag	480
aaggacctgc tggctgaggc cggacgacag cagaaggagg tcgaggacaa catcaagcga	540
ctggaggtgg agatccaggc ccaggagctg aaggccaacg acctgcaggc cgagctggag	600
gaggtggagc agcaggaggc ctctaaggtc gtcaagggca agaccgccgg caaggtgaac	660
gtgctggctg gcctcgccaa gtctcgagtg gaggagctgc gaaacgccct gatggacgtg	720
cgaaaggagc gagatgacac ccgagccga gttaagggaac tcgaagagat cctgtctaag	780
ttcaagggtg agtacaaccc caacttcaac gacgagggag tgaagcgagc cgtgcgatct	840
tgggaggact acgcccga gggcaccctg gagggcgccg tgaacaacgc ccaggaccga	900
gacctggacg agatcgccaa gcccgacgac gagaaggccg gcatcaactg ggagcagtgg	960
gagaacgagg aggacggctg tgaggctggc ctggtgtacc agctggccgc ctacctgcc	1020
ccctctctgg tggagtctat cgagggcaag gtgctgttcg tgcgaggcct gctggaggac	1080

-continued

---

```

aacggcatcc tgcccaaggc cgccgagacc tctacctctg agtctaaggt ggtgtctgag 1140
gcccagagg ccgtaagtc tgccgagaag gagctgggag acaagcagaa gcagctgaag 1200
gaccacaagt ctgacctgga gaccgactac ggcgtgggct ctatcttcag agccctgaag 1260
ggcgtgtgta tctctaagga ctctggcgag tacacctacg agcactgttt tctggaccag 1320
accaagcaga tccccaagaa gggcgaggc tctacccgaa tgggcaagta caccggcatc 1380
ggctctgtgt ctgtggacgt gctgaacgag gccggcgaga tcgtgcctga ggatcgagt 1440
accctgcagt acgccaacgg ccagggtgtg tggaacggac ccgcccagtc taccaccgtg 1500
atcctgacct gtggcgagga ggacgccatc ctgaagggtg ccgaggacga gaagtgtgtg 1560
tactctatgc acgtgacctc tcccgccgtg tgtcccgagc gtgacgaggg cgccaccgcc 1620
cccaaccgaa aggacgagct gtaatagcct a 1651

```

```

<210> SEQ ID NO 9
<211> LENGTH: 1534
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetically generated oligonucleotide

```

```

<400> SEQUENCE: 9

```

```

gagctcagcg ctaccaagcg aggtctctcc aacccccccc gagccgcgcg tgtgaaggcc 60
gccttcacga cctcttgaa cgctaccac cacttcgcct tccccacga cgacctgcac 120
cccgtgtcta actcgttcga cgacgagcga aacggctggg gctcttctgc catcgacggc 180
ctggacacgg ccctcctgat gggcgacgcc gacatcgtga acaccatcct gcagtacgtg 240
ccccagatca acttcaccac caccgcctg gccaaaccagg gcctctctgt gttcgagacc 300
aacatccgat acctgggcgg cctgctgtct gcctacgacc tgctgcgagg ccccttctct 360
tctctggcca ccaaccagac cctggtgaac tctctgctgc gacaggccca gacctggcc 420
aacggcctga aggtggcttt taccaccccc tctggcgtgc ccgacccac cgtgttcttc 480
aacccccagg tgcgacgac tggcgcctct tctaacaacg tggccgagat cggtctctctg 540
gtgctggagt ggacccgact gcttgacctg accggcaacc ccagtagcgc ccagctggcc 600
cagaagggcg agtcttacct gctgaacccc aagggtcttc ccgaggcctg gcccgactg 660
atcgacacct tcgtgtctac ctctaacggc accttcacgg actcttcagg ctcttggtct 720
ggcctgatgg actctttcta cgagtacctg atcaagatgt acctgtacga ccccgaggcc 780
ttcgcccaact acaaggaccg atgggtgctg gccgcgact ctaccatcgc ccacctggcc 840
tctcacccct ctacccgaaa ggacctgacc ttctgtcct cttacaacgg ccagtctacc 900
tctcccaact ctggacacct ggttccttc gccggtggca acttcacct gggcggcac 960
ctgctgaacg agcagaagta catcgacttc ggcataagc tggcctcttc ctacttcgcc 1020
acctacaacc agaccgcctc tggcatcgcc ccgagggtc tcgcctgggt ggactctgtg 1080
accggcgtg gcggtctcc cccctcttct cagtctggtc tctactcttc tgcggcttc 1140
tgggtgacgg cccctacta catcctgcga ccgagaccc tggagtctct gtactacgcc 1200
taccgagtga ccggcgactc taagtggcag gacctggcct gggaggcctt ctctgccatc 1260
gaggacgcct gtcgagccgg ctctgcctac tcttctatca acgacgtgac ccaggccaac 1320
ggtggcgagg cctctgacga catggagtct ttctggttcg ccgaggccct gaagtacgcc 1380
tacctgatct tcgccgagga atctgacgtg cagggtgcagg ccaacggcgg caacaagttc 1440

```

-continued

---

gtgttcaaca ccgaggccca ccccttctct atccgatctt cttctcgacg aggcggccac	1500
ctggcccatg acgagctgta atagcctagg tacc	1534

<210> SEQ ID NO 10  
 <211> LENGTH: 500  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Exemplary nucleotide sequence for the GAP promoter of *Yarrowia lipolytica*

<400> SEQUENCE: 10

cgggcgactg cgtccgaacc agtccagca gcgttttttc cgggccattg agccgactgc	60
gaccccgcca acgtgtcttg gccacgcac tcatgtcatg ttggtgttg gagggcactt	120
tttaagtagc acaaggcacc tagctcgag caaggtgtcc gaaccaaaga agcggctgca	180
gtggtgcaaa cggggcgga acggcggaa aaagccacgg gggcacgaat tgaggcacgc	240
cctcgaattt gagacgagtc acggcccat tcgcccgcgc aatggctcgc caacgcccg	300
tcttttgac cacatcaggt taccacaagc caaaccttg tggtaaaaag cttaacatat	360
tataccgaac gtaggtttg gcgggcttg tccgtctgtc caaggcaaca ttatataag	420
ggtctgcac gccggctcaa ttgaatctt tttctcttc tctctctat attcattctt	480
gaattaaaca cacatcaaca	500

<210> SEQ ID NO 11  
 <211> LENGTH: 6549  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Exemplary motif

<400> SEQUENCE: 11

cttatctggg gcagtgaagt atatgttatg gtaatagtta cgagttagtt gaacttatag	60
atagactgga ctatacggct atcgggtcaa attagaaaga acgtcaatgg ctctctgggc	120
ggaattcgta taacttcgta tagcaggagt tatccgaagc gataattacc ctgttatccc	180
tagcttatcg atacgcgtgc atgctgaggt gtctcacaag tgccgtgcag tcccccccc	240
acttgcttct ctttgtgtgt agtgtaagta cattatcgag accgttggtc ccgccacct	300
cgatccggca tgctgagggt tctcacaagt gccgtgcagt cccgccccca cttgcttctc	360
tttgtgtgta gtgtacgtac attatcgaga ccgttggtcc cgcccacctc gatccggcat	420
gctgagggtg ctcacaagt cgtgcagtc ccgccccac ttgcttctct ttgtgtgtag	480
tgtacgtaca ttatcgagac cgttggtccc gccacctcg atccggcatg ctgaggtgtc	540
tcacaagtgc cgtgcagtc cgccccact tgettctctt tgtgtgtagt gtacgtacat	600
tatcgagacc gttgttccc cccacctga tccggcatgc actgatcacg ggcaaaagt	660
cgtatatata caagagcgtt tgccagccac agattttcac tccacacacc acatcacaca	720
tacaaccaca cacatccagc tgggaacccg aaactaagga tccatgaagc ttccaccat	780
cctcttcaca gcctgcgcta cctggcgcg taccaagcga ggctctccca accccacccg	840
agccgcgct gtgaaggcgc cttccagac ctcttggaac gcctaccacc acttcgcctt	900
ccccacgac gacctgcacc ccgtgtctaa ctcgttcgac gacgagcga acggctgggg	960
ctctctgcc atcgacggcc tggacaccgc catcctgatg ggcgacgcg acatcgtgaa	1020
caccatcctg cagtacgtgc ccagatcaa cttcaccacc accgccgtgg ccaaccagg	1080

-continued

---

catctctgtg	ttcgagacca	acatccgata	cctgggcggc	ctgtgtctg	cctacgacct	1140
gtgcgcaggc	cccttctctt	ctctggccac	caaccagacc	ctggtgaact	ctctgtctgc	1200
acaggcccag	acctggcca	acggcctgaa	ggtggctttt	accacccct	ctggcgtgcc	1260
cgacccccacc	gtgttcttca	acccccacct	gcgacgatct	ggcgectctt	ctaacaacgt	1320
ggccgagatc	ggctctctgg	tgctggagtg	gacccgactg	tctgacctga	cgggcaaccc	1380
ccagtacgcc	cagctggccc	agaaggcgga	gtcttacctg	ctgaacccca	agggtctctc	1440
cgaggcctgg	cccggactga	tcggcacctt	cgtgtctacc	tctaaccgca	ccttcagga	1500
ctcttcgggc	tcttggctg	gctgatgga	ctctttctac	gagtacctga	tcaagatgta	1560
cctgtacgac	cccgtggcct	tcgcccacta	caaggaccga	tgggtgctgg	cgcgcgactc	1620
taccatcgcc	cacctggcct	ctcaccctc	taccgaaaag	gacctgacct	tcctgtctc	1680
ttacaacggc	cagtctacct	ctcccaactc	tggacacctg	gcttccttcg	cgggtggcaa	1740
cttcatectg	ggcggcatcc	tgctgaacga	gcagaagtac	atcgacttcg	gcataaagct	1800
ggcctcttcc	tacttcgcca	cctacaacca	gaccgcctct	ggcatcgcc	ccgagggctt	1860
cgcctgggtg	gactctgtga	cggcgctgg	cggctctccc	ccctctctc	agtctggctt	1920
ctactcttct	gccggcttct	gggtgaccgc	cccctactac	atcctgcgac	ccgagacct	1980
ggagtctctg	tactacgct	accgagtga	cggcgactct	aagtggcagg	acctggcctg	2040
ggaggccttc	tctgccatcg	aggacgcctg	tcgagccggc	tctgcctact	cttctatcaa	2100
cgactgacc	caggccaaag	gtggcggagc	ctctgacgac	atggagtctt	tctggttcgc	2160
cgaggccctg	aagtacgct	acctgatctt	cgccgaggaa	tctgacgtgc	aggtgcaggc	2220
caacggcggc	aacaagtctg	tggtcaaac	cgaggcccac	cccttctcta	tccgatcttc	2280
ttctcgacga	ggcggccacc	tggcccatga	cgagctgtaa	tagcctaggg	tgtctgtggt	2340
atctaagcta	tttatcactc	tttacaactt	ctacctcaac	tatctacttt	aataaatgaa	2400
tatcgtttat	tctctatgat	tactgtatat	cgttccctct	aagacaaatc	gaattccatg	2460
tgtaaacactc	gctctggaga	gttagtcac	cgacagggtg	actctaactc	cccaacacct	2520
tattaaactct	gcgtaactgt	aactcttctt	gccacgtcga	tcttactcaa	tttctctgct	2580
catcatctgc	tggattgttg	tctatcgtct	ggctctaata	catttattgt	ttattgccca	2640
aacaactttc	attgcacgta	agtgaattgt	tttataacag	cgttcgcca	ttgctgcgcc	2700
atcgtcgtcc	ggctgtccta	ccgttaggg	agtgtgtctc	acactaccga	ggttactaga	2760
gttgggaaag	cgatactgcc	tcggacacac	cacctggctt	tacgactgca	gagagaatcg	2820
gcgttacctc	ctcacaaagc	cctcagtgcg	gccgcccggg	gtgggcgaag	aactccagca	2880
tgagatcccc	gcgctggagg	atcatccagc	cggcgtcccc	gaaaacgatt	ccgaagccca	2940
acctttcata	gaaggcgggc	gtggaatcga	aatctcgtga	tggcaggttg	ggcgtcgctt	3000
ggtcggctcat	ttcgaacccc	agagtccgc	tcagaagaac	tcgtcaagaa	ggcgatagaa	3060
ggcgatgcgc	tgcaatccgg	gagcggcgat	accgtaaagc	acgaggaagc	ggtcagccca	3120
ttcgcgcgca	agctcttcag	caatatcag	ggtagccaac	gctatgtcct	gatagcggtc	3180
cgccacaccc	agccggccac	agtcgatgaa	tccagaaaag	cggccatttt	ccaccatgat	3240
attcggcaag	caggcatcgc	catgggtcac	gacgagatcc	tcgccgtcgg	gcagcgcgcc	3300
cttgagcctg	gcgaacagtt	cggctggcgc	gagccctga	tgtctctcgt	ccagatcacc	3360
ctgatcgaca	agaccggctt	ccatccgagt	acgtgctcgc	tcgatgcgat	gtttcgcttg	3420
gtggtcgaat	gggcaggtag	ccggatcaag	cgtatgcagc	cgcgcatttg	catcagccat	3480

-continued

---

gatggatact	ttctcggcag	gagcaagggtg	agatgacagg	agatcctgcc	cgggcacttc	3540
gcccataagc	agccagtcce	ttcccgttc	agtgacaacg	tcgagcacag	ctgcgcaagg	3600
aacgcccgtc	gtggccagcc	acgatagccg	cgctgcctcg	tcctgcagtt	cattcagggc	3660
accggacagg	tcggtcttga	caaaaagaac	cgggcgcccc	tgcgctgaca	gccggaacac	3720
ggcggcatca	gagcagccga	ttgtctgttg	tgcccagtc	tagccgaata	gcctctccac	3780
ccaagcggcc	ggagaacctg	cgtgcaatcc	atcttggtca	atcatgcgaa	acgatcctca	3840
tcctgtctct	tgatcagatc	ttgatccct	gcgccatcag	atccttggtg	gcaagaaagc	3900
catccagttt	actttgcagg	gcttcccaac	cttaccagag	ggcgccccag	ctggcaattc	3960
cgggttcgctt	gctgtccata	aaaccgccc	gtctagctat	cgccatgtaa	gccactgca	4020
agctacctgc	tttctctttg	cgttgcggtt	ttcccttgtc	cagatagccc	agtagctgac	4080
attcatccgg	ggtcagcacc	gtttctgcgg	actggcttcc	tacgtgttcc	gcttctctta	4140
gcagcccttg	cgcctgaggt	gcttgccgca	gcgtgaagct	agcttatgct	gtgtgaaata	4200
ccgcacagat	gcgtaaggag	aaaataccgc	atcaggcgct	cttccgcttc	ctcgctcact	4260
gactcgctgc	gctcggctgt	tcggctgcgg	cgagcgggat	cagctcactc	aaaggcggta	4320
atacgggttat	ccacagaatc	aggggataac	gcaggaaaga	acatgtgagc	aaaaggccag	4380
caaaaggcca	ggaaccgtaa	aaaggccgcg	ttgctggcgt	ttttccatag	gtcccgcccc	4440
cctgacgagc	atcacaaaaa	tcgacgctca	agtcagaggt	ggcgaaaccc	gacaggacta	4500
taaagatacc	aggcggttcc	ccctggaagc	tcctcgtgc	gctctcctgt	tccgaccctg	4560
ccgcttacgg	gatacctgtc	cgcctttctc	ccttcgggaa	gcgtggcgct	ttctcatagc	4620
tcacgctgta	ggatctctag	ttcgggtgag	gtcgttcgct	ccaagctggg	ctgtgtgcac	4680
gaaccccccg	ttcagcccca	ccgctgcgcc	ttatccggt	actatcgctc	tgagtccaac	4740
ccggtaaagc	acgacttata	gccactggca	gcagccactg	gtaacaggat	tagcagagcg	4800
aggatatgtg	gcggtgtctc	agagttcttg	aagtgggtgg	ctaactacgg	ctaacactaga	4860
aggacagtat	ttggtatctg	cgtctctgtg	aagccagtta	ccttcggaaa	aagagttggt	4920
agctcttgat	ccggcaaaac	aaccaccgct	ggtagcggcg	gttttttggt	tgcaagcagc	4980
agattacgct	cagaaaaaaa	ggatctcaag	aagatccttt	gatcttttct	tactgaacgg	5040
tgatccccac	cggaattgct	gccgctgtcg	ggaaccgcgt	tcagggtgaa	caggacacct	5100
cccttgcact	tcttggtata	tcagtatagg	ctgatgtatt	catagtgggg	tttttcataa	5160
taaatttact	aacggcaggc	aacattcact	cggcttaaac	gcaaaacgga	ccgtcttgat	5220
atcttctgac	gcattgacca	ccgagaaata	gtgttagtta	ccgggtgagt	tattgttctt	5280
ctacacaggc	gacgcccata	gtctagagtt	gatgtactaa	ctcagatttc	actacctacc	5340
ctatccctgg	tacgcacaaa	gcactttgct	agatagagtc	gacaaaggcg	ggccccccct	5400
cgagattacc	ctgttatccc	tacataactt	cgtatagcat	acattatacg	aagttattct	5460
gaattccgag	aaacacaaca	acatgcccc	ttggacagac	catgcggata	cacaggttgt	5520
gcagtaccat	acatactoga	tcagacaggt	cgtctgacca	tcatacaagc	tgaacagcgc	5580
tccatacttg	cacgctctct	atatacacag	ttaaattaca	tatccatagt	ctaacctcta	5640
acagttaatc	ttctggtaag	cctcccagcc	agccttctgg	tatcgcttgg	cctcctcaat	5700
aggatctcgg	ttctggcgtg	acagacctcg	gccgacaatt	atgatatccg	ttccggtaga	5760
catgacatcc	tcaacagttc	ggtaactgctg	tccgagagcg	tctcccttgt	cgtcaagacc	5820

-continued

---

caccccgggg gtcagaataa gccagtcctc agagtcgccc ttaggtcggt tctgggcaat	5880
gaagccaacc acaaaactcg ggctcgatcg ggcaagctca atggctctgt tggagtactc	5940
gccagtggcc agagagccct tgcaagacag ctcggccagc atgagcagac ctctggccag	6000
cttctcgttg ggagagggga ctaggaaactc cttgtactgg gagttctcgt agtcagagac	6060
gtcctccttc ttctgttcag agacagtttc ctcggcacca gctcgcaggc cagcaatgat	6120
tccggttcgg ggtacacggt gggcggttgt gatatcgac cactcgcgca ttcggtgaca	6180
ccggtactgg tgcttgacag tgttgccaat atctgcgaac tttctgtcct cgaacaggaa	6240
gaaaccgtgc ttaagagcaa gtcccttgag ggggagcaca gtgccggcgt aggtgaagtc	6300
gtcaatgatg tcgatatggg tcttgatcat gcacacataa ggcccgacct tatcggaag	6360
ctcaatgagc tccttggttg tggtaacatc cagagaagca cacaggttgg tttcttggc	6420
tgccacgagc ttgagcactc gagcggcaaa ggcggacttg tggacgttag ctcgagcttc	6480
gtaggagggc attttggttg tgaagaggag actgaaataa atttagtctg cagaactttt	6540
tatcggaac	6549

&lt;210&gt; SEQ ID NO 12

&lt;211&gt; LENGTH: 6485

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Exemplary motif

&lt;400&gt; SEQUENCE: 12

tagcaggagt tatccgaagc gataattacc ctgttatccc tagcttatcg atacggcgga	60
ctgcgtccga accagctcca gcagcgtttt ttccgggcca ttgagccgac tgcgaccccc	120
ccaacgtgtc ttggcccacg cactcatgtc atgttggtgt tgggaggcca ctttttaagt	180
agcacaaagg acctagctcg cagcaagggt tccgaaccaa agaagcggct gcagtgggtc	240
aaacggggcg gaaacggcgg gaaaaagcca cgggggcacg aattgaggca cgcctcga	300
tttgagacga gtcacggccc cattcgcccg cgcaatggct cgccaacgcc cggctctttg	360
caccacatca ggttaccca agccaaacct ttgtgttaaa aagcttaaca tattataccg	420
aacgtagggt tgggggggct tgctccgtct gtccaaggca acatttatat aagggtctgc	480
atcgccggct caattgaatc ttttttcttc ttctcttctc tatattcatt cttgaattaa	540
acacacatca acaggatcca tgaagctttc caccatcctc ttcaagcct gcgctaccct	600
ggccgctacc aagcgaggct ctcccaaccc caccgagcc gccgctgtga aggccgcctt	660
ccagacctct tggaaagcct accaccactt cgccttcccc cagcagacc tgcaccccg	720
gtctaactcg ttcgacgacg agcgaacgg ctggggctct tctgccatcg acggcctgga	780
caccgccatc ctgatgggag acgccgacat cgtgaacacc atcctgcagt acgtgcccc	840
gatcaacttc accaccacgg ccgtggccaa ccagggcacg tctgtgttcg agaccaacat	900
ccgatacctg ggccgctgc tgtctgcta cgacctgtg cgaggccctt tctcttctct	960
ggccaccaac cagaccctgg tgaactctct gctgcgacag gccagaccc tggccaacgg	1020
cctgaagggt gcttttaacca cccctctggt cgtgcccagc cccaccgtgt tcttcaaccc	1080
caccgtgcga cgatctggcg cctcttctaa caacgtggcc gagatcggct ctctggtgct	1140
ggagtggacc cgactgtctg acctgaccgg caacccccag tacgcccagc tggcccagaa	1200
ggcgagctct tacctgtctg accccaaggg ctctcccagc gcctggcccg gactgatcgg	1260
caccttcgtg tctaccteta acggcacctt ccaggactct tccggctctt ggtctggcct	1320

-continued

---

gatggactct	ttctacgagt	acctgatcaa	gatgtacctg	tacgaccccg	tggccttcgc	1380
ccactacaag	gaccgatggg	tgttggecgc	cgactctacc	atcgcccacc	tggcctctca	1440
cccctctacc	cgaaggacc	tgaccttctt	gtcctcttac	aacggccagt	ctacctctcc	1500
caactctgga	cacctggctt	ccttcgccgg	tggcaacttc	atcctgggcg	gcatectgct	1560
gaacgagcag	aagtacatcg	acttcggcat	caagctggcc	tcttctact	tcgccaccta	1620
caaccagacc	gcctctggca	tcggcccccga	gggcttcgcc	tgggtggact	ctgtgaccgg	1680
cgctggcggc	tctccccct	cttctcagtc	tggcttctac	tcttctgccg	gcttctgggt	1740
gaccgcccc	tactacatcc	tgcgaccga	gaccttgag	tctctgtact	acgcctaccg	1800
agtgaccggc	gactctaagt	ggcaggacct	ggcctgggag	gccttctctg	ccatcgagga	1860
cgctgtcga	gccggtctg	cctactcttc	tatcaacgac	gtgaccagg	ccaacggtag	1920
cggagcctct	gacgacatgg	agtctttctg	gttcgccgag	gccctgaagt	acgcctacct	1980
gatcttcgcc	gaggaatctg	acgtgcaggt	gcaggccaac	ggcggaaca	agttcgtgtt	2040
caacaccgag	gccccccct	tctctatccg	atcttctctt	cgacgaggcg	gccacctggc	2100
ccatgacgag	ctgtaatagc	ctagggtgtc	tgtggtatct	aagctattta	tcactcttta	2160
caacttctac	ctcaactatc	tactttaata	aatgaatata	gtttattctc	tatgattact	2220
gtatatgctg	tcctctaaga	caaatcgaat	tccatgtgta	acactcgctc	tggagagtta	2280
gtcatccgac	agggtaaact	taatctccca	acaccttatt	aactctgcgt	aactgtaact	2340
cttcttgcca	cgctgatctt	actcaatttt	cctgctcacc	atctgctgga	ttgttgctta	2400
tcgtctggct	ctaatacatt	tattgtttat	tgcctaaaca	actttcattg	cacgtaagtg	2460
aattgtttta	taacagcggt	cgccaattgc	tgcgccatcg	tcgtccggct	gtcctaccgt	2520
tagggtagtg	tgtctcacac	taccgagggt	actagagttg	ggaaagcgat	actgcctcgg	2580
acacaccacc	tggctcttac	actgcagaga	gaatcggcgt	tacctcctca	caaagccctc	2640
agtgccggcg	cccggggtgg	gcgaagaact	ccagcatgag	atccccgcgc	tggaggatca	2700
tccagccggc	gtccccgaaa	acgattccga	agcccaacct	ttcatagaag	gcggcggtgg	2760
aatcgaaatc	tcgtgatggc	aggttgggcg	tcgcttggtc	ggtcatttcg	aacccagag	2820
tcccgtcag	aagaactcgt	caagaaggcg	atagaaggcg	atgcgctgcg	aatcgggagc	2880
ggcgataccg	taaagcacga	ggaagcggtc	agcccatcgc	ccgccaagct	cttcagcaat	2940
atcacgggta	gccaacgcta	tgtcctgata	gcggctccgc	acaccagcc	ggccacagtc	3000
gatgaatcca	gaaaagcggc	cattttccac	catgatattc	ggcaagcagg	catcgccatg	3060
ggtcacgacg	agatcctcgc	cgtcgggcgt	gcgcgccttg	agcctggcga	acagttcggc	3120
tggcgcgagc	ccctgatgct	cttcgtccag	atcctcctga	tcgacaagac	cggtctccat	3180
ccgagtacgt	gctcgctcga	tcgcatgttt	cgttggtggg	tcgaatgggc	aggtagccgg	3240
atcaagcgta	tgcagccgcc	gcattgcata	agccatgatg	gatactttct	cggcaggagc	3300
aaggtagatg	gacaggagat	cctgcccccg	cacttcgccc	aatagcagcc	agtccttctc	3360
cgcttcagtg	acaacgtcga	gcacagctgc	gcaaggaaag	cccgtcgtgg	ccagccacga	3420
tagccgcgct	gcctcgtcct	gcagttcatt	cagggcaccg	gacaggtcgg	tcttgacaaa	3480
aagaaccggg	cgccccctgc	ctgacagccg	gaacacggcg	gcacagagc	agccgattgt	3540
ctgttggtgc	cagtcatagc	cgaatagcct	ctccacccaa	gcggccggag	aacctgcgtg	3600
caatccatct	tgttcaatca	tcgaaaaaga	tcctcctcct	gtctcttgat	cagatcttga	3660

-continued

---

tccccctgcgc	catcagatcc	ttggcggcaa	gaaagccatc	cagtttactt	tgcagggett	3720
cccaacctta	ccagagggcg	ccccagctgg	caattccggt	tcgcttgctg	tcataaaac	3780
cgcccagctc	agctatcgcc	atgtaagccc	actgcaagct	acctgctttc	tctttgcgct	3840
tgcgttttcc	cttgctcaga	tagcccagta	gctgacattc	atccgggggc	agcaccgttt	3900
ctgcggactg	gctttctacg	tgttccgctt	ccttttagcag	cccttgcgcc	ctgagtgcct	3960
gcggcagcgt	gaagctagct	tatgcgggtg	gaaataccgc	acagatgcgt	aaggagaaaa	4020
taccgcatca	ggcgccttcc	cgcttcctcg	ctcactgact	cgctgcgctc	ggtcggtcgg	4080
ctgcggcgag	cgggtatcagc	tcactcaaag	gcggtaatac	ggttatccac	agaatcaggg	4140
gataacgcag	gaaagaacat	gtgagcaaaa	ggccagcaaa	aggccaggaa	ccgtaaaaag	4200
gccgcgttgc	tggcggtttt	ccataggttc	cgccccctg	acgagcatca	caaaaatcga	4260
cgctcaagtc	agaggtggcg	aaacccgaca	ggactataaa	gataaccaggc	gtttccccct	4320
ggaagctccc	tcgtgcgctc	tctgttccg	accctgccgc	ttaccggata	cctgtccgcc	4380
tttctccctt	cgggaagcgt	ggcgctttct	catagctcac	gctgtaggta	tctcagttcg	4440
gtgtaggctg	ttcgctccaa	gctgggctgt	gtgcacgaac	cccccgctca	gcccgaccgc	4500
tgcgccttat	ccggtaaacta	tcgtcttgag	tccaacccgg	taagacacga	cttatcgcca	4560
ctggcagcag	ccactggtaa	caggattagc	agagcgaggt	atgtaggcgg	tgctacagag	4620
ttcttgaagt	gggtggcctaa	ctacggctac	actagaagga	cagtatttgg	tatctgcgct	4680
ctgctgaagc	cagttacott	cggaaaaaga	gttggtagct	cttgatccgg	caaaaaaac	4740
accgctggta	gcggcggttt	tttgtttgca	agcagcagat	tacgcgcaga	aaaaaaggat	4800
ctcaagaaga	tcctttgatc	ttttcttact	gaacgggtgat	ccccaccgga	attgcggccg	4860
ctgtcgggaa	ccgcgttcag	gtggaacagg	acacctccct	tgcacttctt	ggtatatcag	4920
tataggctga	tgtattcata	gtggggtttt	tcataataaa	tttactaacg	gcaggcaaca	4980
ttcactcggc	ttaaacgcaa	aacggaccgt	cttgatatct	tctgacgcac	tgaccaccga	5040
gaaatagtgt	tagttaccgg	gtgagttatt	gttcttctac	acaggcgacg	cccatcgtct	5100
agagttgatg	tactaactca	gatttcacta	cctaccctat	ccctggtacg	cacaaagcac	5160
tttgctagat	agagtcgaca	aaggcggggc	ccccctcgag	attaccctgt	tatccctaca	5220
taacttcgta	tagcatacat	tatacgaagt	tattctgaat	tccgcccaga	gagccattga	5280
cgttctttct	aatttggaac	gatagccgta	tagtccagtc	tatctataag	ttcaactaac	5340
tcgtaactat	taccataaca	tatacttcac	tgccccagat	aaggttccga	taaaaagttc	5400
tgcagactaa	atttatttca	gtctctctct	caccacaaaa	atgcctcctc	acgaagctcg	5460
agctaacgtc	cacaagtcgg	cctttgccgc	tcgagtgcct	aagctcgtgg	cagccaagaa	5520
aaccaacctg	tgtgcttctc	tggatgttac	caccaccaag	gagctcattg	agcttgccga	5580
taaggtcgga	ccttatgtgt	gcatgatcaa	gaccatatac	gacatcattg	acgacttcac	5640
ctacgccggc	actgtgctcc	ccctcaagga	acttgcctct	aagcacgggt	tcttctgtt	5700
cgaggacaga	aagttcgag	atattggcaa	cactgtcaag	caccagtacc	ggtgtcaccg	5760
aatcgccgag	tggtcggata	tcaccaacgc	ccacgggtgta	cccggaaccg	gaatcattgc	5820
tggcctcgga	gctggtgccc	aggaaactgt	ctctgaacag	aagaaggagg	acgtctctga	5880
ctacgagaac	tcccagtaca	aggagtccct	agtcctctct	cccaacgaga	agctggccag	5940
aggtctgctc	atgctggccg	agctgtcttg	caagggtctc	ctggccactg	gcgagtactc	6000
caagcagacc	attgagcttg	cccgatccga	ccccgagttt	gtggttggtt	tcattgcca	6060

-continued

---

```

gaaccgacct aagggcgact ctgaggactg gcttattctg acccccgggg tgggtcttga 6120
cgacaagggg gacgctctcg gacagcagta ccgaactgtt gaggatgtca tgtctaccgg 6180
aacggatata ataattgtcg gccgaggtct gtacggccag aaccgagatc ctattgagga 6240
ggccaagcga taccagaagg ctggctggga ggcttaccag aagattaact gttagaggtt 6300
agactatgga tatgtaattt aactgtgtat atagagagcg tgcaagtatg gacgctgtt 6360
cagcttgtat gatggtcaga cgacctgtct gatcgagtat gtatggtact gcacaacctg 6420
tgtatccgca tggctgtgcc aatggggcat gttgttgtgt ttctcggaat tcgtataact 6480
tcgta 6485

```

```

<210> SEQ ID NO 13
<211> LENGTH: 6996
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Exemplary motif

```

```

<400> SEQUENCE: 13

```

```

caaggggtag gcgaatggta cgattccgcc aagtgagact ggcgatcggg agaagggttg 60
gtggtcattg gggatagaat ttgtacaagt ggaaaaacca ctacgagtag cggatttgat 120
accacaagta gcagagatat acagcaatgg tgggagtgcg agtatcggaa tgtactgtac 180
ctcctgtact cgtactcgta cggcactcgt agaaacgggg caatacgggg gagaagcgat 240
cgcccgctcg ttcaatcgcc acaagtccga gtaatgctcg agtatcgaag tcttgtacct 300
ccctgtcaat catggcacca ctgggtcttga cttgtctatt catactggac aagcgccaga 360
gttagctagc gaatttcgcc ctccgacatc accccatacg acggacacac atgcccgaca 420
aacagcctct cttattgtag ctgaaagtat attgaatgtg aacgtgtaca atatcaggta 480
ccagcgggag gttacggcca aggtgatacc ggaataaccc tggcttggag atggtcggtc 540
cattgtactg aagtgtccgt gtcgtttccg tcaactgccc aattggacat gtttgttttt 600
ccgatcttcc gggcgccctc tccttgtctc cttgtctgtc tcctggactg ttgctacccc 660
attttcttgg cctccattgg ttccctcccg tctttcacgt cgtctatggt tgcattggtt 720
cccttatact tttcccaca gtcacatgtt atggaggggt ctagatggac atggtgcaag 780
gcccgcaggg ttgattcgac gcttttcgcg gaaaaaaca agtccaaata ccccggttta 840
ttctccctcg gctctcggtt ttccacatga aaactataac ctagactaca cgggcaacct 900
taaccccaga gtatacttat ataccaaagg gatgggtcct caaaaatcac acaagcaacg 960
gatccatgaa gctttccacc atcctcttca cagcctgcgc taccttggcc gctaccaagc 1020
gaggtctctc caaccccacc cgagccgcgc ctgtgaaggc cgccttccag acctcttgga 1080
acgcctacca ccatttcgcc ttccccacg acgacctgca ccccggtgtc aactcgttcg 1140
acgacgagcg aaacggctgg ggctcttctg ccatcgacgg cctggacacc gccatcctga 1200
tgggcgacgc cgacatcgtg aacaccatcc tgcagtacgt gcccagatc aacttcacca 1260
ccacgcgcgt ggccaaccag ggcatctctg tgttcgagac caacatccga tacctgggcg 1320
gcctgctgtc tgccctacgc ctgctgcgag gcccttctc ttctctggcc accaaccaga 1380
ccctgggtgaa ctctctgctg cgacaggccc agaccctggc caacggcctg aaggtggctt 1440
ttaccacccc ctctggcgctg ccgcacccca ccgtgttctt caaccccacc gtgcgacgat 1500
ctggcgccctc ttctaacaac gtggccgaga tcggctctct ggtgctggag tggaccgcac 1560

```

-continued

---

tgtctgacct	gaccggcaac	ccccagtagc	cccagctggc	ccagaagggc	gagtcttacc	1620
tgctgaaccc	caagggtctc	cccgaggcct	ggcccgact	gacgggcacc	ttcgtgtcta	1680
cctctaaccg	caccttccag	gactcttccg	gctcttggtc	tggcctgatg	gactctttct	1740
acgagtacct	gatcaagatg	tacctgtacg	accccggtggc	cttcgcccac	tacaaggacc	1800
gatgggtgct	ggccgcccac	tctaccatcg	cccacctggc	ctctcaccac	tctaccggaa	1860
aggacctgac	cttctgtgcc	tcttacaacg	gccagtctac	ctctcccaac	tctggacacc	1920
tggcttcctt	cgccggtggc	aacttcaccc	tgggcggcat	cctgtgtaac	gagcagaagt	1980
acatcgactt	cggcatcaag	ctggcctctt	cctacttcgc	cacctacaac	cagaccgcct	2040
ctggcatcgg	ccccgagggc	ttcgctggg	tggactctgt	gaccggcgct	ggcggtcttc	2100
ccccctcttc	tcagtctggc	ttctactctt	ctgccggctt	ctgggtgacc	gccccctact	2160
acatcctgcg	acccgagacc	ctggagtctc	tgtactacgc	ctaccgagtg	accggcgact	2220
ctaagtggca	ggacctggcc	tgggaggcct	tctctgccat	cgaggacgcc	tgtcgagccg	2280
gctctgccta	ctcttctatc	aacgacgtga	cccaggccaa	cgggtggcgga	gcctctgacg	2340
acatggagtc	tttctggttc	gccgagggcc	tgaagtacgc	ctacctgac	ttcgccgagg	2400
aatctgacgt	gcagggtgcg	gccaacggcg	gcaacaagtt	cgtgttcaac	accgagggcc	2460
accccttctc	tatccgatct	tcttctcgac	gaggcgccca	cctggcccat	gacgagctgt	2520
aatagcctag	ggtgtctgtg	gtatctaagc	tatttatcac	tctttacaac	ttctacctca	2580
actatctact	ttaataaatg	aatatcggtt	attctctatg	attactgtat	atgcgttcct	2640
ctaagacaaa	tcgaattcca	tgtgtaaac	tcgctctgga	gagttagtca	tcgacaggg	2700
taactcta	ctcccaacac	cttattaact	ctgcgtaact	gtaactcttc	ttgccacgtc	2760
gatcttactc	aattttctctg	ctcatcatct	gctggattgt	tgtctatcgt	ctggctctaa	2820
tacatttatt	gtttattgac	caaacaactt	tcattgcacg	taagtgaatt	gttttataac	2880
agcgttcgcc	aattgctgcg	ccatcgctgt	cgggctgtcc	taccgttagg	gtagtgtgtc	2940
tcacactacc	gaggttacta	gagttgggaa	agcgatactg	cctcgacac	accacctggt	3000
cttacgactg	cagagagaat	cggcggtacc	tcctcacaaa	gccctcagtg	cggccgccc	3060
gggtgggcga	agaactccag	catgagatcc	cgcgctgga	ggatcatcca	gccggcgctc	3120
cggaaaacga	ttccgaagcc	caacctttca	tagaaggcgg	cgggtggaatc	gaaatctcgt	3180
gatggcaggt	tgggcgtcgc	ttggtcggtc	atttcgaacc	ccagagtccc	gctcagaaga	3240
actcgtcaag	aaggcgatag	aaggcgatgc	gctgcgaatc	gggagcggcg	ataccgtaaa	3300
gcacgaggaa	gcggtcagcc	cattcgccgc	caagctcttc	agcaatatca	cgggtagcca	3360
acgctatgtc	ctgatagcgg	tccgccacac	ccagccggcc	acagtcgatg	aatccagaaa	3420
agcggccatt	ttccaccatg	atattcggca	agcaggcatc	gccatgggtc	acgacgagat	3480
cctcgccgtc	gggcatcgcc	gccttgagcc	tggcgaacag	ttcggtggc	gcgagcccct	3540
gatgctcttc	gtccagatca	tctgatcga	caagaccggc	ttccatccga	gtacgtgttc	3600
gctcgatcgc	atgtttcgct	tgggtggtcga	atgggcaggt	agccggatca	agcgtatgca	3660
gccgccgcat	tgcacagcc	atgatggata	ctttctcgcc	aggagcaagg	tgagatgaca	3720
ggagatcctg	ccccggcact	tcgcccata	gcagccagtc	ccttcccgtc	tcagtgacaa	3780
cgtcgagcac	agctgcgcaa	ggaacgccc	tcgtggccag	ccacgatagc	cgcgctgcct	3840
cgtcctgcag	ttcattcagg	gcaccggaca	ggtcggctct	gacaaaaaga	accgggcgcc	3900
cctgcgctga	cagccggaac	acggcgccat	cagagcagcc	gattgtctgt	tgtgcccagt	3960

-continued

---

catagccgaa tagcctctcc acccaagcgg cgggagaacc tgcgtgcaat ccatcttggt	4020
caatcatgcg aaacgatcct cactctgtct cttgatcaga tcttgatccc ctgcgccatc	4080
agatccttgg cggcaagaaa gccatccagt ttactttgca gggcttccca accttaccag	4140
agggcgcccc agctggcaat tccggttcgc ttgctgtcca taaaaccgcc cagtctagct	4200
atcgccatgt aagcccactg caagctacct gctttctctt tgcgcttgcg ttttcccttg	4260
tccagatagc ccagtagctg acattcatcc ggggtcagca ccgtttctgc ggactggctt	4320
tctacgtggt ccgcttcott tagcagccct tgcgcctga gtgcttgagg cagcgtgaag	4380
ctagcttatg cgggtgtgaaa taccgcacag atgcgtaagg agaaaatacc gcatacggcg	4440
ctcttcgctt tcctcgctca ctgactcgct gcgctcggtc gttcggctgc ggcgagcggg	4500
atcagctcac tcaaaggcgg taatacgggt atccacagaa tcaggggata acgcaggaaa	4560
gaacatgtga gcaaaaggcc agcaaaaggc caggaaaccgt aaaaaggccg cgttgctggc	4620
gtttttccat aggtctcgcc ccctgacga gcatcacaaa aatcgacgct caagtcagag	4680
gtggcgaaac ccgacaggac tataaagata ccaggcggtt cccctggaa gctccctcgt	4740
gcgctctcct gttccgaccc tgcgcttac cggatacctg tccgccttcc tcccttcggg	4800
aagcgtggcg ctttctcata gctcacgctg taggtatctc agttcgggtg aggtcgttcg	4860
ctccaagctg ggctgtgtgc acgaaccccc cgttcagccc gaccgctgcg ccttatccgg	4920
taactatcgt cttgagtcca acccgtaag acacgactta tcgccactgg cagcagccac	4980
tggtaacagg attagcagag cgaggatatg aggcgggtgt acagagttct tgaagtgggtg	5040
gcctaactac ggctacacta gaaggacagt atttggtatc tgcgctctgc tgaagccagt	5100
taccttcgga aaaagagttg gtactctctg atccggcaaa caaacaccgc ctggtagcgg	5160
cgggtttttg tttgcaagca gcagattacg cgcagaaaaa aaggatctca agaagatcct	5220
ttgatctttt cttactgaac ggtgatcccc accggaattg cggccgctgt cgggaaccgc	5280
gttcagggtg aacaggacac ctcccttgca cttcttggtg tatcagtata ggctgatgta	5340
ttcatagtgg ggtttttcat aataaattta ctaacggcag gcaacattca ctcggtctaa	5400
acgcaaaaac gaccgtcttg atatctctcg acgcattgac caccgagaaa tagtgttagt	5460
taccgggtga gttattgttc ttctacacag gcgacgccc tcgtctagag ttgatgtact	5520
aactcagatt tcactaccta cctatccct ggtacgcaca aagcactttg ctagatagag	5580
tcgacaaaag cgggcccccc ctgcagatta cctgtttatc cctacataac ttctgatagc	5640
atacattata cgaagttatt ctgaattccg ccagagagc cattgacgtt ctttctaatt	5700
tggaccgata gccgtatagt ccagctctac tataagtcca actaactcgt aactattacc	5760
ataacatata cttcactgcc ccagataagg ttccgataaa agtttctgca gactaaattt	5820
atttcagtct cctcttcacc accaaaatgc cctcctacga agctcgagct aacgtccaca	5880
agtccgcctt tgcgctcga gtgctcaagc tcgtggcagc caagaaaacc aacctgtgtg	5940
cttctctgga tgtaaccacc accaaggagc tcattgagct tgcgataag gtccgacctt	6000
atgtgtgcat gatcaagacc catatcgaca tcattgacga cttcacctac gccggcactg	6060
tgtccccctc caaggaaact gctcttaagc acggtttctt cctgttcgag gacagaaagt	6120
tcgcagatat tggcaacact gtcaagcacc agtaccggtg tcaccgaatc gccgagtggg	6180
ccgatatcac caacgcccac ggtgtacccg gaaccggaat cattgctggc ctgcgagctg	6240
gtgccgagga aactgtctct gaacagaaga aggaggacgt ctctgactac gagaactccc	6300

-continued

---

```

agtacaagga gttcctagtc cctctctcca acgagaagct ggccagaggt ctgctcatgc 6360
tggccgagct gtcttgcaag ggctctctgg ccaactggcg gtactccaag cagaccattg 6420
agcttgcccc atccgacccc gagtttgtgg ttggcttcat tgcccagaac cgacctaaag 6480
gcgactctga ggactggctt attctgaccc ccgggggtggg tcttgacgac aaggagagacg 6540
ctctcggaca gcagtaccga actgttgagg atgtcatgtc taccggaacg gatatacataa 6600
ttgtcgcccg aggtctgtac ggcagaacc gagatcctat tgaggaggcc aagcgatacc 6660
agaaggctgg ctgggaggct taccagaaga ttaactgtta gaggttagac tatggatatg 6720
taatttaact gtgtatatag agagcgtgca agtatggagc gctgttcagc ttgtatgatg 6780
gtcagacgac ctgtctgacg gagtatgtat ggtactgcac aacctgtgta tccgcatggt 6840
ctgtccaatg gggcatgttg ttgtgtttct cggaattcgt ataacttcgt atagcaggag 6900
ttatccgaag cgataattac cctgttatcc cttagcttacc gattoccaca agacgaacaa 6960
gtgataggcc gagagccgag gacgaggtgg agtgca 6996

```

&lt;210&gt; SEQ ID NO 14

&lt;211&gt; LENGTH: 7195

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Exemplary motif

&lt;400&gt; SEQUENCE: 14

```

taattaccct gttatcccta gcttatcgat agagaccggg ttggcgcggt atttgtgtcc 60
caaaaaacag cccaattgc cccaattgac cccaaattga ccagtagcg ggcccaaccc 120
cggcgagagc ccccttcacc ccacatatca aacctccccc gggtcccaca cttgcggtta 180
agggcgtagg gtactgcagt ctggaatcta cgcttggtca gactttgtac tagtttcttt 240
gtctggccat ccgggtaacc catgccggac gcaaaataga ctactgaaa tttttttgct 300
ttgtggttgg gactttagcc aagggtataa aagaccacgg tcccgaatt acctttcctc 360
ttctttttctc tctctccttg tcaactcaca cccgaaggat ccatgaagct ttccaccatc 420
ctcttcacag cctgcgtac cctggcgct accaagcgag gctctccaa cccaccgca 480
gcccgcgctg tgaaggcgcg ctccagacc tcttggaacg cctaccacca cttcgccctc 540
ccccacgacg acctgcaccc cgtgtctaac tcgttcgacg acgagcgaaa cggtcggggc 600
tcttctgcca tcgacggcct ggacacggcc atcctgatgg gcgacggcga catcgtgaac 660
accatcctgc agtacgtgcc ccagatcaac ttcaccacca ccgccgtggc caaccagggc 720
atctctgtgt tcgagaccaa catccgatac ctggggcgcc tgctgtctgc ctacgacctg 780
ctgcgaggcc ccttctcttc tctggccacc aaccagaccc tgggtgaactc tctgctgcga 840
caggcccaga ccctggccaa cggcctgaag gtggctttta ccacccctc tggcgtgccc 900
gacccccacg tgttcttcaa cccaccgtg cgacgatctg gcgcctcttc taacaacgtg 960
gccgagatcg gctctctggt gctggagtgg acccgactgt ctgacctgac cggaaccccc 1020
cagtacgccc agctggccca gaaggcgag tcttacctgc tgaaccccaa gggtctctcc 1080
gaggcctggc ccggactgat cggcaccttc gtgtctacct ctaacggcac cttccaggac 1140
tcttcgggct cttggtctgg cctgatggac tctttctacg agtacctgat caagatgtac 1200
ctgtacgacc ccgtggcctt cgcacctac aaggaccgat ggggtgctggc cgccgactct 1260
accatcgccc acctggcctc tcacctctc acccgaaagg acctgacctt cctgtcctct 1320
tacaacggcc agtctacctc tcccaactct ggacacctgg cttccttcgc cggtggcaac 1380

```

-continued

---

ttcatcctgg	gcggcatoct	gctgaacgag	cagaagtaca	tcgaactcgg	catcaagctg	1440
gcctcttctc	actteggccac	ctacaaccag	accgcctctg	gcateggccc	cgagggettcc	1500
gcctgggtgg	actctgtgac	cgcgctggc	ggctctcccc	cctcttctca	gtctggettcc	1560
tactcttctg	ccggttctg	ggtagccgc	ccctactaca	tcctgcgacc	cgagaccctg	1620
gagtctctgt	actacgccta	ccgagtgacc	ggcgactcta	agtggcagga	cctggcctgg	1680
gaggccttct	ctgccatcga	ggacgcctgt	cgagccggct	ctgcctactc	ttctatcaac	1740
gacgtgaccc	aggccaacgg	tggcggagcc	tctgacgaca	tggagtcttt	ctggttcgcc	1800
gaggccctga	agtacgccta	cctgatcttc	gccgaggaat	ctgacgtgca	ggtgcaggcc	1860
aacggcggca	acaagtctgt	gttcaacacc	gaggccccacc	ccttctctat	ccgatcttct	1920
tctcgacgag	gcgcccaoct	ggcccatgac	gagctgtaat	agcctagggt	gtctgtggta	1980
tctaagctat	ttatcactct	ttacaacttc	tacctcaact	atctacttta	ataaatgaat	2040
atcgtttatt	ctctatgatt	actgtatatg	cgttcctcta	agacaaatcg	aattccatgt	2100
gtaacactcg	ctctggagag	ttagtcatcc	gacagggtaa	ctctaacttc	ccaacacctt	2160
attaactctg	cgtaactgta	actcttcttg	ccacgtcgat	cttactcaat	tttctgctc	2220
atcatctgct	ggattgttgt	ctatcgcttg	gctctaatac	atctattgtt	tattgccccaa	2280
acaactttca	ttgcacgtaa	gtgaattgtt	ttataacagc	gttcgccaat	tgtgcgccca	2340
tcgtcgtccg	gctgtcctac	cgtaggggta	gtgtgtctca	cactaccgag	gttactagag	2400
ttgggaaagc	gatactgcct	cggacacacc	acctggctct	acgactgcag	agagaatcgg	2460
cgttacctcc	tcacaaagcc	ctcagtgcgg	ccgcgccggg	tgggcgaaga	actccagcat	2520
gagatccccg	cgctggagga	tcattccagcc	ggcgtccccg	aaaacgattc	cgaagcccaa	2580
cctttcatag	aaggcggcgg	tggaaatcgaa	atctcgtgat	ggcagggttg	gcgtcgcttg	2640
gtcggtcatt	tcgaacccca	gagtccccgt	cagaagaact	cgtaagaag	gcgatagaag	2700
gcgatgcgt	gcgaatcggg	agcggcgata	ccgtaaagca	cgagggaagc	gtcagcccat	2760
tcgccgccaa	gctcttcagc	aatatcacgg	gtagccaaag	ctatgtcctg	atagcgggtcc	2820
gccacaccca	gccggccaca	gtcgatgaat	ccagaaaagc	ggccattttc	caccatgata	2880
ttcggcaagc	aggcatcgcc	atgggtcacg	acgagatcct	cgccgtcggg	catgcgcgcc	2940
ttgagcctgg	cgaacagttc	ggctggcgcg	agccccgat	gctcttcgtc	cagatcatcc	3000
tgatcgacaa	gaccggcttc	catccgagta	cgtgctcgct	cgatgcgatg	tttcgcttgg	3060
tggtcgaatg	ggcaggtagc	cgatcaagc	gtatgcagcc	gccgcattgc	atcagccatg	3120
atggatactt	tctcggcagg	agcaagggtga	gatgacagga	gatcctgccc	cggcacttctg	3180
cccaatagca	gccagtccct	tcccgttca	gtgacaacgt	cgagcacagc	tgcgcaagga	3240
acgcccgtcg	tggccagcca	cgatagccgc	gctgcctcgt	cctgcagttc	attcagggca	3300
ccggacaggt	cgttcttgac	aaaaagaacc	gggcgccccct	gcgctgacag	ccggaacacg	3360
ggggcatcag	agcagccgat	tgtctgttgt	gccagtcatt	agccgaatag	cctctccacc	3420
caagcggcgg	gagaacctgc	gtgcaatcca	tcttgttcaa	tcattgcgaaa	cgatcctcat	3480
cctgtctctt	gatcagatct	tgatccccctg	cgccatcaga	tccttgccgg	caagaaagcc	3540
atccagttta	ctttgcaggg	cttcccaacc	ttaccagagg	gcgccccagc	tggaatttcc	3600
ggttcgcttg	ctgtccataa	aaccgcccag	tctagctatc	gccatgtaag	cccactgcaa	3660
gctacctgct	ttctctttgc	gcttgcggtt	tcccttgctc	agatagccca	gtagctgaca	3720

-continued

---

ttcatccggg gtcagcaccg tttctgcgga ctggctttct acgtgttccg cttectttag	3780
cagcccttgc gccctgagtg cttgcggcag cgtgaagcta gcttatgcgg tgtgaaatac	3840
cgcacagatg cgtaaggaga aaataccgca tcaggcgctc ttcgcttcc tcgctcactg	3900
actcgctcgc ctcggtcggt cggtcgcggc gagcggatc agctcactca aaggcggtaa	3960
tacggttatc cacagaatca ggggataacg caggaaagaa catgtgagca aaaggccagc	4020
aaaaggccag gaaccgtaaa aaggccgctg tgctggcggt tttccatagg ctccgcccc	4080
ctgacgagca tcacaaaaat cgacgctcaa gtcagaggtg gcgaaacccg acaggactat	4140
aaagatacca ggcgtttccc cctggaagct cctcgtcgcg ctctcctgtt ccgacccctgc	4200
cgcttaccgg atacctgtcc gccctttctcc cttegggaag cgtggcgctt tctcatagct	4260
cacgctgtag gtatctcagt tcggtgtagg tcgttcgctc caagctgggc tgtgtgcacg	4320
aacccccctg tcagcccgac cgctgcgcct tatccggtaa ctatcgtctt gagtccaacc	4380
cggtaagaca cgacttatcg ccactggcag cagccactgg taacaggatt agcagagcga	4440
ggatatgtagg cgggtgtaca gagttcttga agtggtggcc taactacggc taaactagaa	4500
ggacagtatt tggtatctgc gctctgctga agccagttac ctteggaaaa agagttggta	4560
gctcttgatc cggcaaaaca accaccgtg gtacggcgcg ttttttgttt gcaagcagca	4620
gattacgcgc agaaaaaaag gatctcaaga agatccttg atcttttctt actgaacggt	4680
gatccccacc ggaattgcgg ccgctgtcgg gaaccgcgtt caggtggaac aggacacctc	4740
ccttgcaatt cttggtatat cagtataggc tgatgtatcc atagtggggt ttttcataat	4800
aaatttacta cacaccacct ggtcttacga ctgcagagag aatcggcggt acctcctcac	4860
aaagccctca gtgcggcgcc ccgggggtgg cgaagaactc cagcatgaga tccccgcgct	4920
ggaggatcat ccagccggcg tcccggaaaa cgattccgaa gccaacctt tcatagaagg	4980
cggcgggtga atcgaaatct cgtgatggca ggttgggcgt cgcttggtcg gtcatttcga	5040
accccgaggt cccgctcaga agaactcgtc aagaaggcga tagaaggcga tgcgctgcga	5100
atcgggagcg gcgataccgt aaagcacgag gaagcggtea gccattcgc cgccaagctc	5160
ttcagcaata tcacgggtag ccaacgctat gtctgatag cggtecgcca caccagccg	5220
gccacagtgc atgaatccag aaaagcggcc attttccacc atgatattcg gcaagcaggc	5280
atcgccatgg gtcacgacga gatcctcgcc gtcgggcatg cgcgccttga gcctggcgaa	5340
cagttcggct ggcgcgagcc cctgatgctc ttcgtccaga tcactcctgat cgacaagacc	5400
ggcttccatc cgagtacgtg ctgcgtcgat gcgatgttcc gcttggtggt cgaatgggca	5460
ggtagccgga tcaagcgtat gcagccgcgg cattgcacca gccatgatgg atactttctc	5520
ggcaggagca aggtgagatg acaggagatc ctgccccggc acttcgcccc atagcagcca	5580
gtcccttccc gcttcagtga caacgtcgag cacagctcgc caagggaacgc ccgtcgtggc	5640
cagccacgat agccgcgctg cctcgtcctg cagttcattc agggcaccgg acaggtcggt	5700
cttgacaaaa agaaccgggc gccctcgcgc tgacagccgg aacacggcgg catcagagca	5760
gccgattgtc tggtgtgccc agtcatagcc gaatagcctc tccaccaag cggccggaga	5820
acctgcgtgc aatccatctt gttcaatcat gcgaaacgat cctcatcctg tctcttgatc	5880
agatcttgat cccctgcgcc atcagatcct tggcggcaag aaagccatcc agtttacttt	5940
gcagggtctc ccaaccttac cagagggcgc ccagctggc aattccgggt cgettgctgt	6000
ccataaaacc gccagtccta gctatcgcca tgtaagccca ctgcaagcta cctgctttct	6060
ctttgcgctt gcgttttccc ttgtccagat agcccagtag ctgacattca tccggggtea	6120

-continued

---

```

gcaccgtttc tgcggactgg ctttctacgt gttecgcttc ctttagcagc cettgcgccc 6180
tgagtgtctg cggcagcgtg aagctagctt atgcgggtgtg aaataccgca cagatgcgta 6240
aggagaaaaat accgcatcag gcgctcttcc gcttcctcgc tcaactgactc gctgcgctcg 6300
gtcgttcggc tgcggcgagc ggtatcagct cactcaaagg cggtaatagc gttatccaca 6360
gaatcagggg ataacgcagg aaagaacatg tgagcaaaa gccagcaaaa ggccagggaac 6420
cgtaaaaagg ccgcgttgct ggcggttttc cataggtccc gccccctga cgagcatcac 6480
aaaaatcgac gctcaagtca gaggtggcga aaccgcagc gactataaag ataccaggcg 6540
tttccccctg gaagctccct cgtgcgctct cctgttccga ccctgccgct taccggatac 6600
ctgtccgcct ttctcccttc ggaagcgtg gcgctttctc atagctcacg ctgtagggat 6660
ctcagttcgg tgtaggctgt tcgctccaag ctgggctgtg tgcacgaacc ccccgttcag 6720
cccgaccgct gcgccttata cggttaactat cgtcttgagt ccaaccgggt aagacacgac 6780
ttatcgccac tggcagcagc cactggtaac aggattagca gagcgaggta tgtaggcggt 6840
gctacagagt tcttgaagtg gtggcctaac tacggctaca ctagaaggac agtatttggt 6900
atctgcgctc tgctgaagcc agttaccttc ggaaaaagag ttggtagctc ttgatccggc 6960
aaacaaacca ccgctggtag cgcggtttt ttgtttgcaa gcagcagatt acgcgcagaa 7020
aaaaaggatc tcaagaagat cttttgatct ttcttactg aacggtgatc cccaccggaa 7080
ttgcggccgc tgcgggaac cgcgttcagg tgaacagga cacctccctt gcacttcttg 7140
gtatatcagt ataggctgat gtattcatag tggggttttt cataataaat ttact 7195

```

```

<210> SEQ ID NO 15
<211> LENGTH: 1518
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetically generated oligonucleotide

<400> SEQUENCE: 15

```

```

gagctcagcg ctcgaccctg tatccccaag tctttcggt actcttctgt ggtgtgtgtg 60
tgtaacgcc cctactgtga ctcggtcgac cccccacac tccccgccc gggcaccttc 120
tctcgatacg agtctaccg atctggccga cgaatggagc tgtctatggg ccccatccag 180
gccaaaccaca ccggcaccgg cctgctgctg accctgcagc ccgagcagaa gttccagaag 240
gtcaagggct tcggcggagc catgaccgac gccgctgccc tgaacatcct ggccctgtct 300
cccccgctc agaacctgct cctgaagtct tacttctctg aggaggcat cggtacaaac 360
atcatccgag tgccatggc ctcttgtagc ttctctatcc gaacctacac ctacgccgac 420
acccccgacg acttccagct gcacaacttc tccctgcccc aggaggacac caagctgaag 480
atccccctga tccaccgagc cctgcagctg gccagcgac ccgtgtctct gctggcctct 540
ccctggacct ctcccactg gctcaagacc aacggcgccg tgaacggcaa gggctctctg 600
aagggccagc ccggcgacat ctaccaccag acctggccc gatacttcgt gaagttcctg 660
gacgcctacg ccgagcacia gctgcagttc tgggcccgtga ccgcccagaa cgagccctct 720
gctggactgc tgtccggeta ccccttcagc tgtctgggct tcacccccga gcaccagcga 780
gacttcacg cccgagacct gggaccacac ctggccaact ctaccacca caacgtgcga 840
ctgctgatgc tggacgacca gcgactgctg ctgccccact gggccaaggt ggtgctgacc 900
gaccccgagg ccgccaagta cgtccacggc atcgctgtcc attggtatct ggactttctg 960

```

-continued

---

gctcccgcca aggccaccct gggcgagacc caccgactgt tccccaacac catgctgttc	1020
gcctctgagg cttgtgtggg ctctaagttc tgggagcagt ctgtgcgact gggctcttgg	1080
gaccgaggca tgcagtactc tcactctatc atcaccaacc tgetgtacca cgtggtgggc	1140
tggaccgact ggaacctggc cctgaacccc gagggcggac ccaactgggt gcgaaacttc	1200
gtggactctc ccatcatcgt ggacatcacc aaggacacct tctacaagca gccatgttc	1260
taccacctgg gccacttctc taagtctc cccgagggt ctcagcgagt gggcctggtg	1320
gcctctcaga agaacgacct ggacgcctg gccctgatgc accccgacgg ctctgccgtg	1380
gtggtgtgtec tgaaccgatc ttctaaggac gtgcccctga ccatcaagga cccgcctg	1440
ggcttctctg agaccatctc tcccggctac tctatccaca cctacctgtg gcgacgacag	1500
taatagccta ggggtacc	1518

<210> SEQ ID NO 16  
 <211> LENGTH: 498  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetically generated oligonucleotide

<400> SEQUENCE: 16

gctcccccc gactgatctg tgactctcga gtgctggaac gatacctgct ggaagccaag	60
gaagccgaga acatcaccac cggtctgtgc gagcactgtt ctctgaacga gaacattacc	120
gtgcccgaca ccaaggtgaa cttctacgcc tgggaagcga tggaggtggg ccagcaggcc	180
gtggaggtgt ggcagggact ggctctgctg tctgagggcg tgctgcgagg acaggctctc	240
ctggtgaact cttctcagcc ctgggagccc ctgcagctgc acgtggacaa ggccgtgtct	300
ggcctgcgat ctctgaccac cctgctgcga gccctcgggt ctcagaagga agccatctct	360
cccccgacg ccgcctctgc tgccccctg cgaaccatca ccgcccacac cttccgaaag	420
ctgttcgag tgtactctaa cttcctgcga ggcaagctga agctgtacac cggcgggct	480
tgtcgaaccg gcgaccga	498

<210> SEQ ID NO 17  
 <211> LENGTH: 1194  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetically generated oligonucleotide

<400> SEQUENCE: 17

ctggacaaag gcctggcccg aacccccacc atgggctggc tgcactggga gcgattcatg	60
tgtaacctgg actgtcagga agagcccgac tcttgtatct ctgagaagct gttcatggaa	120
atggccgagc tgatggtgtc tgagggtgg aaggacgcg gctacgagta cctgtgtatc	180
gacgactgtt ggatggcccc ccagcgagac tctgagggcc gactccaggc cgacccccag	240
cgattcccc acggcatccg acagctcgcc aactacgtgc actctaaggc cctgaagctg	300
ggcatctacg ccgacgtggg caacaagacc tgtgcggct tccccgctc ttocgctac	360
tacgacatcg acgcccagac cttcgccgac tggggcgtgg acctgctgaa gttcgacggc	420
tgttactgtg actctctcga gaacctggcc gacggctaca agcacatgtc tctggccctg	480
aaccgaaccg gccgatctat cgtgtactct tgtgagtggc ccctgtacat gtggcccttc	540
cagaagccca actacaccga gatccgacag tactgttaacc actggcgaaa cttcgccgac	600
atcgacgact cgtggaagtc tatcaagtct attctggact ggacctctt caaccaggag	660

-continued

---

cgaatcgctg acgtcgccgg acccgcgga tggaacgacc cgcacatgct ggtgatcggc	720
aacttcggcc tgtcttgga ccagcagggtg acccagatgg cctgtgggc tatcatggct	780
gccccctgt tcatgtctaa cgacctgca cacatctctc cccaggccaa ggccctgctc	840
caggacaagg acgtgatgc catcaaccag gacccctgg gcaagcagg ctaccagctc	900
cgacaggcg acaacttcga ggtgtgggag cgacccctgt ctggcctggc ctgggccgtg	960
gccatgatca accgacagga gatcgcgga ccccgatctt acaccatgc cgtggcctcc	1020
ctgggaaagg gcgtggcctg taaccccgcc tgtttcatca cccagctcct gcccgtaag	1080
cgaaagctgg gattctacga gtggacctct cgactgcgat ctacatcaa cccaccggc	1140
accgtgctgc tccagctcga gaacaccatg cagatgtctc tgaaggacct gctg	1194

<210> SEQ ID NO 18  
 <211> LENGTH: 19  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Primer

<400> SEQUENCE: 18

tcgctatcac gtctctagc	19
----------------------	----

<210> SEQ ID NO 19  
 <211> LENGTH: 23  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Primer

<400> SEQUENCE: 19

tctctgtata cttgtatgta ctg	23
---------------------------	----

<210> SEQ ID NO 20  
 <211> LENGTH: 38  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Primer

<400> SEQUENCE: 20

ctagggataa cagggtaatg ggtgtacgaa gtatcgag	38
---	----

<210> SEQ ID NO 21  
 <211> LENGTH: 36  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Primer

<400> SEQUENCE: 21

cattaccctg ttatccctag cgagatcatg gactgg	36
---	----

<210> SEQ ID NO 22  
 <211> LENGTH: 34  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Primer

<400> SEQUENCE: 22

gacgcggcgc catgagcttc aacattocca aaac	34
---------------------------------------	----

-continued

---

<210> SEQ ID NO 23  
<211> LENGTH: 49  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer  
  
<400> SEQUENCE: 23  
  
ctagggataa cagggtaata caaaattcag aaataaaaat actttacag 49  
  
<210> SEQ ID NO 24  
<211> LENGTH: 39  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer  
  
<400> SEQUENCE: 24  
  
cattaccctg ttatccctaa gtaacatgag tgctatgag 39  
  
<210> SEQ ID NO 25  
<211> LENGTH: 30  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer  
  
<400> SEQUENCE: 25  
  
cgcttaatta aatgcatgga ggtattgctg 30  
  
<210> SEQ ID NO 26  
<211> LENGTH: 46  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer  
  
<400> SEQUENCE: 26  
  
ggtgcttcga ctatcagttt cggaggattg ggtgattctt tttatg 46  
  
<210> SEQ ID NO 27  
<211> LENGTH: 46  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer  
  
<400> SEQUENCE: 27  
  
cataaaaaga atcacccaat cctccgaaac tgatagtcga agcacc 46  
  
<210> SEQ ID NO 28  
<211> LENGTH: 32  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer  
  
<400> SEQUENCE: 28  
  
tgagcggcgg cttttctact tcagagctgg ag 32  
  
<210> SEQ ID NO 29  
<211> LENGTH: 34  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer

-continued

---

<400> SEQUENCE: 29

ggcttaatta attggtagtg atataatgta acgc

34

<210> SEQ ID NO 30

<211> LENGTH: 39

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 30

tagggataac agggtaatca cgacacatac tcattcaag

39

<210> SEQ ID NO 31

<211> LENGTH: 37

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 31

attaccctgt tatccctaga aggagatgta gcgtaag

37

<210> SEQ ID NO 32

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 32

tgataaatag cttagatacc acag

24

<210> SEQ ID NO 33

<211> LENGTH: 19

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 33

acatacaacc acacacatc

19

<210> SEQ ID NO 34

<211> LENGTH: 28

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 34

ggcggatcca tggtgctgca cccgtttc

28

<210> SEQ ID NO 35

<211> LENGTH: 31

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 35

ggccctaggc tactcaaact cctcgcaat c

31

<210> SEQ ID NO 36

-continued

<211> LENGTH: 27  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 36

ggtctcgcca gcgcgccac cctcttc

27

<210> SEQ ID NO 37  
<211> LENGTH: 28  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 37

ctagatcagc aataaagtcg tgctgggc

28

<210> SEQ ID NO 38  
<211> LENGTH: 30  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 38

ggatccatgt ctatcaagcg agaagagtc

30

<210> SEQ ID NO 39  
<211> LENGTH: 34  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 39

cctaggctag atcagcaata aagtcgtgct gggc

34

<210> SEQ ID NO 40  
<211> LENGTH: 41  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Synthetically generated oligonucleotide

<400> SEQUENCE: 40

cgactatccg gttcggatca ttgggtgatt ctttttatga g

41

<210> SEQ ID NO 41  
<211> LENGTH: 41  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Synthetically generated oligonucleotide

<400> SEQUENCE: 41

ctcataaaaa gaatcaccca atgatccgaa ccggatagtc g

41

<210> SEQ ID NO 42  
<211> LENGTH: 41  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Synthetically generated oligonucleotide

<400> SEQUENCE: 42

-continued

---

cgactatccg gttcggatca ggtgggtgatt ctttttatga g 41

<210> SEQ ID NO 43  
 <211> LENGTH: 41  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetically generated oligonucleotide  
 <400> SEQUENCE: 43

ctcataaaaa gaatcaccac ctgatccgaa ccggatagtc g 41

<210> SEQ ID NO 44  
 <211> LENGTH: 46  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetically generated oligonucleotide  
 <400> SEQUENCE: 44

ggtgcttcga ctatcagttt cggaggattg ggtgattctt tttatg 46

<210> SEQ ID NO 45  
 <211> LENGTH: 46  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetically generated oligonucleotide  
 <400> SEQUENCE: 45

cataaaaaga atcacccaat cctccgaaac tgatagtcga agcacc 46

<210> SEQ ID NO 46  
 <211> LENGTH: 38  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetically generated oligonucleotide  
 <400> SEQUENCE: 46

cccgatatcg gatccatgaa gaactctgtc ggtatttc 38

<210> SEQ ID NO 47  
 <211> LENGTH: 70  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetically generated oligonucleotide  
 <400> SEQUENCE: 47

gggaagctta acgcggttcc agcgggtccg gatacggcac cggcgcaccc aacgaccaac 60

ctgtggtcag 70

<210> SEQ ID NO 48  
 <211> LENGTH: 36  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetically generated oligonucleotide  
 <400> SEQUENCE: 48

cagtgcggcc gcactccctc ttttactca ctattg 36

<210> SEQ ID NO 49

-continued

---

<211> LENGTH: 46  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Synthetically generated oligonucleotide

<400> SEQUENCE: 49

cattaccctg ttatccctac gctcagatcc aattgttttg gtggtc 46

<210> SEQ ID NO 50  
<211> LENGTH: 53  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Synthetically generated oligonucleotide

<400> SEQUENCE: 50

gtagggataa cagggtaatg ctctcaagga cggaccagat gagactgtta tcg 53

<210> SEQ ID NO 51  
<211> LENGTH: 46  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Synthetically generated oligonucleotide

<400> SEQUENCE: 51

gactttaatt aaaccctatg tggcacctca acccacatct cccgtc 46

<210> SEQ ID NO 52  
<211> LENGTH: 35  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Synthetically generated oligonucleotide

<400> SEQUENCE: 52

cagtggatcc atgaactctc ctattttcac taccg 35

<210> SEQ ID NO 53  
<211> LENGTH: 35  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Synthetically generated oligonucleotide

<400> SEQUENCE: 53

gactcctagg aagcttccag gttacaagtt gttac 35

<210> SEQ ID NO 54  
<211> LENGTH: 73  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 54

cctaggtcac tccaatcccc caaacagggt gctgacgctc gactcatagt gagctagatc 60

agcaataaag tcg 73

<210> SEQ ID NO 55  
<211> LENGTH: 30  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer

-continued

---

<400> SEQUENCE: 55

ggatccatgt ctatcaagcg agaagagtc

30

<210> SEQ ID NO 56

<211> LENGTH: 25

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 56

gaattcatgc ccgtagattc ttctc

25

<210> SEQ ID NO 57

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 57

gagtcttcg gaggattcag

20

<210> SEQ ID NO 58

<211> LENGTH: 19

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 58

cctggaagaa taaaaagtc

19

<210> SEQ ID NO 59

<211> LENGTH: 30

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 59

cctaggctat tcctggaaga atacaaagtc

30

<210> SEQ ID NO 60

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 60

ggtattgctg agcgtatgca aa

22

<210> SEQ ID NO 61

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 61

ccaccgatcc atacggagta ct

22

<210> SEQ ID NO 62

-continued

---

```

<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 62

cgacctggaa tctgcacttc aa                22

<210> SEQ ID NO 63
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 63

cggtagccacc taaggcttcc aa                22

<210> SEQ ID NO 64
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 64

ccagccaact gtgttgattc aa                22

<210> SEQ ID NO 65
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 65

ggagctgggtg gaataccagt ca                22

<210> SEQ ID NO 66
<211> LENGTH: 57
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 66

gatccatgaa gctttccacc atcctcttca cagcctgcgc taccctggcc gcggtac        57

<210> SEQ ID NO 67
<211> LENGTH: 65
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 67

gtaccggcgc gccgcttctg gagaactgcg gcctcagaag gagtgatggg ggaagggagg        60

gcggc                                         65

<210> SEQ ID NO 68
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

```

-continued

&lt;400&gt; SEQUENCE: 68

ggcagcgcta caaaacgtgg atctcccaac

30

&lt;210&gt; SEQ ID NO 69

&lt;211&gt; LENGTH: 30

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Primer

&lt;400&gt; SEQUENCE: 69

ggccctaggt tacaactcgt cgtgagcaag

30

&lt;210&gt; SEQ ID NO 70

&lt;211&gt; LENGTH: 17

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Pichia pastoris

&lt;400&gt; SEQUENCE: 70

atccagcagt gatgacg

17

&lt;210&gt; SEQ ID NO 71

&lt;211&gt; LENGTH: 17

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Saccharomyces cerevisiae

&lt;400&gt; SEQUENCE: 71

atccagccgt gattacg

17

&lt;210&gt; SEQ ID NO 72

&lt;211&gt; LENGTH: 15

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Pichia pastoris

&lt;400&gt; SEQUENCE: 72

ggtctgcagc accat

15

&lt;210&gt; SEQ ID NO 73

&lt;211&gt; LENGTH: 15

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Saccharomyces cerevisiae

&lt;400&gt; SEQUENCE: 73

tgtccgaagc gcagt

15

&lt;210&gt; SEQ ID NO 74

&lt;211&gt; LENGTH: 18

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Pichia pastoris

&lt;400&gt; SEQUENCE: 74

Ala Pro Phe Thr Ala Asn Ala Phe Asp Leu Asn Asp Phe Val Phe Phe  
1 5 10 15

Gln Glu

&lt;210&gt; SEQ ID NO 75

&lt;211&gt; LENGTH: 18

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Saccharomyces cerevisiae

&lt;400&gt; SEQUENCE: 75

Glu Ala Gln Ser Gly Leu Asn Ser Phe Glu Leu Asn Asp Phe Phe Ile

-continued

1	5	10	15	
Thr Ser				
<210> SEQ ID NO 76 <211> LENGTH: 63 <212> TYPE: DNA <213> ORGANISM: Artificial Sequence <220> FEATURE: <223> OTHER INFORMATION: synthetically generated oligonucleotides <400> SEQUENCE: 76				
catgaacttt	ccaccatcct	cttcacagcc	tgcgctaccc	60
tggtgccgc	cctcccttcc			
ccc				63
<210> SEQ ID NO 77 <211> LENGTH: 65 <212> TYPE: DNA <213> ORGANISM: Artificial Sequence <220> FEATURE: <223> OTHER INFORMATION: synthetically generated oligonucleotides <400> SEQUENCE: 77				
gtaccggccg	gccgcttctg	gagaactgcg	gcctcagaag	60
gagtgatggg	ggaagggagg			
gcggc				65
<210> SEQ ID NO 78 <211> LENGTH: 57 <212> TYPE: DNA <213> ORGANISM: Artificial Sequence <220> FEATURE: <223> OTHER INFORMATION: synthetically generated oligonucleotides <400> SEQUENCE: 78				
gatccatgaa	gctttccacc	atcctcttca	cagcctgcgc	57
taccctggcc	gcggtac			
<210> SEQ ID NO 79 <211> LENGTH: 57 <212> TYPE: DNA <213> ORGANISM: Artificial Sequence <220> FEATURE: <223> OTHER INFORMATION: synthetically generated oligonucleotides <400> SEQUENCE: 79				
gtacttcgaa	aggtggtagg	agaagtgtcg	gacgcgatgg	57
gaccggcgcc	atggatc			
<210> SEQ ID NO 80 <211> LENGTH: 17 <212> TYPE: DNA <213> ORGANISM: Artificial Sequence <220> FEATURE: <223> OTHER INFORMATION: synthetically generated oligonucleotides <400> SEQUENCE: 80				
gaggctgaag	ccgcgga			17
<210> SEQ ID NO 81 <211> LENGTH: 17 <212> TYPE: DNA <213> ORGANISM: Artificial Sequence <220> FEATURE: <223> OTHER INFORMATION: synthetically generated oligonucleotides				

&lt;400&gt; SEQUENCE: 81

tccgcggett cagcctc

17

What is claimed is:

1. A method of producing an altered N-glycosylation form of a target protein, the method comprising:
  - providing a *Yarrowia lipolytica* or an *Arxula adeninivorans* cell genetically engineered to express an  $\alpha$ -1,2-mannosidase polypeptide; and
  - introducing into the cell a nucleic acid encoding a target protein, wherein the cell produces the target protein in an altered N-glycosylation form.
2. The method of claim 1, further comprising isolating the altered N-glycosylation form of the target protein.
3. The method of claim 1, wherein the target protein is an exogenous protein.
4. The method of claim 1, wherein the target protein is an endogenous protein.
5. The method of claim 1, wherein the target protein is a mammalian protein.
6. The method of claim 1, wherein the target protein is a pathogen protein, a lysosomal protein, a growth factor, a cytokine, a chemokine, or a fusion protein.
7. The method of claim 1, wherein the target protein is a protein associated with a lysosomal storage disorder (LSD).
8. The method of claim 7, wherein the lysosomal storage disorder is Gaucher disease, Tay-Sachs disease, Pompe disease, Niemann-Pick disease, or Fabry disease.
9. The method of claim 7, wherein the target protein is selected from the group consisting of glucocerebrosidase, alpha-galactosidase, galactocerebrosidase, alpha-L-iduronidase, beta-D-galactosidase, beta-glucosidase, beta-hexosaminidase, beta-D-mannosidase, alpha-L-fucosidase, aryl-sulfatase B, arylsulfatase A, alpha-N-acetylglucosaminidase, aspartylglucosaminidase, iduronate-2-sulfatase, alpha-glucosaminide-N-acetyltransferase, beta-D-glucuronidase, hyaluronidase, alpha-L-mannosidase, alphanneuraminidase, phosphotransferase, acid lipase, acid ceramidase, sphingomyelinase, thioesterase, cathepsin K, and lipoprotein lipase.
10. The method of claim 7, wherein the target protein is human alpha-galactosidase A.
11. The method of claim 1, wherein the altered N-glycosylation form comprises one or more N-glycan structures selected from group consisting of Man<sub>5</sub>GlcNAc<sub>2</sub>, Man<sub>8</sub>GlcNAc<sub>2</sub>, and Man<sub>9</sub>GlcNAc<sub>2</sub>.
12. The method of claim 1, wherein the  $\alpha$ -1,2-mannosidase polypeptide is a MNS1 polypeptide.
13. The method of claim 1, wherein the  $\alpha$ -1,2-mannosidase polypeptide is targeted to the endoplasmic reticulum.
14. The method of claim 1, further comprising additional processing of the target protein in its altered N-glycosylation form.
15. The method of claim 14, wherein the additional processing comprises enzymatic or chemical treatment of the altered N-glycosylation form of the target protein.
16. The method of claim 1, wherein the cell is further genetically engineered to comprise at least one additional modification of an N-glycosylation activity.
17. The method of claim 16, wherein the at least one additional modification of an N-glycosylation activity comprises a deficiency in an N-glycosylation activity.
18. The method of claim 16, wherein the at least one additional modification of an N-glycosylation activity comprises expression of a protein having N-glycosylation activity.
19. The method of claim 1, wherein the target protein is a human protein.
20. The method of claim 18, wherein the expressed protein is a polypeptide capable of effecting mannosyl phosphorylation of N-glycans.
21. The method of claim 1, wherein the  $\alpha$ -1,2-mannosidase is the *Saccharomyces cerevisiae* MNS1 polypeptide or the *S. cerevisiae* MNS1 polypeptide comprising one or more amino substitutions selected from the group consisting of R273L, R273G, R269S, and S272G.
22. An isolated *Yarrowia lipolytica* or *Arxula adeninivorans* cell genetically engineered to express an  $\alpha$ -1,2-mannosidase polypeptide, wherein the cell is capable of producing a target protein in an altered glycosylation form if a nucleic acid encoding the target protein is introduced into the cell.
23. The cell of claim 22, said cell further comprising a nucleic acid encoding a target protein, wherein the cell produces the target protein in an altered N-glycosylation form.
24. The cell of claim 22, wherein the cell is further genetically engineered to comprise at least one additional modification of an N-glycosylation activity.
25. The cell of claim 24, wherein the at least one additional modification of an N-glycosylation activity comprises a deficiency in an N-glycosylation activity.
26. The cell of claim 24, wherein the at least one additional modification of an N-glycosylation activity comprises expression of a protein having N-glycosylation activity.
27. The cell of claim 22, wherein the  $\alpha$ -1,2-mannosidase polypeptide is a MNS1 polypeptide.
28. The cell of claim 22, wherein the  $\alpha$ -1,2-mannosidase polypeptide is targeted to the endoplasmic reticulum.
29. The cell of claim 26, wherein the expressed protein is a polypeptide capable of effecting mannosyl phosphorylation of N-glycans.
30. The cell of claim 22, wherein the  $\alpha$ -1,2-mannosidase is the *Saccharomyces cerevisiae* MNS1 polypeptide or the *S. cerevisiae* MNS1 polypeptide comprising one or more amino substitutions selected from the group consisting of R273L, R273G, R269S, and S272G.
31. A method of producing an altered N-glycosylation form of a target protein, the method comprising contacting a target protein with a cell lysate prepared from a *Yarrowia lipolytica* or an *Arxula adeninivorans* cell genetically engineered to express an  $\alpha$ -1,2-mannosidase polypeptide, wherein the contacting of the target protein with the cell lysate results in an altered N-glycosylation form of the target protein.
32. The method of claim 31, wherein the cell is further genetically engineered to comprise at least one additional modification of an N-glycosylation activity.
33. The method of claim 32, wherein the at least one additional modification of an N-glycosylation activity comprises a deficiency in an N-glycosylation activity.

127

34. The method of claim 32, wherein the at least one additional modification of an N-glycosylation activity comprises expression of a protein having N-glycosylation activity.

35. The method of claim 31, wherein the alpha  $\alpha$ -1,2-mannosidase polypeptide is a MNS1 polypeptide.

36. The method of claim 31, wherein the  $\alpha$ -1,2-mannosidase polypeptide is targeted to the endoplasmic reticulum.

37. The method of claim 34, wherein the expressed protein is a polypeptide capable of effecting mannosyl phosphorylation of N-glycans.

38. The method of claim 31, wherein the  $\alpha$ -1,2-mannosidase is the *Saccharomyces cerevisiae* MNS1 polypeptide or the *S. cerevisiae* MNS1 polypeptide comprising one or more amino substitutions selected from the group consisting of R273L, R273G, R269S, and S272G.

39. A substantially pure culture of *Yarrowia lipolytica* or *Arxula adeninivorans* cells, wherein the culture comprises a cell genetically engineered to express an  $\alpha$ -1,2-mannosidase polypeptide, and wherein the cell is capable of producing a target protein in an altered glycosylation form if a nucleic acid encoding the target protein is introduced into the cell.

40. The substantially pure culture of claim 39, said cell further comprising a nucleic acid encoding a target protein, wherein the cell produces the target protein in an altered N-glycosylation form.

128

41. The substantially pure culture of claim 39, wherein the  $\alpha$ -1,2-mannosidase polypeptide is a MNS1 polypeptide.

42. The substantially pure culture of claim 39, wherein the  $\alpha$ -1,2-mannosidase polypeptide is targeted to the endoplasmic reticulum.

43. The substantially pure culture of claim 39, wherein the cell is further genetically engineered to comprise at least one additional modification of an N-glycosylation activity.

44. The substantially pure culture of claim 43, wherein the at least one additional modification of an N-glycosylation activity comprises a deficiency in an N-glycosylation activity.

45. The substantially pure culture of claim 43, wherein the at least one additional modification of an N-glycosylation activity comprises expression of a protein having N-glycosylation activity.

46. The substantially pure culture of claim 45, wherein the expressed protein is a polypeptide capable of effecting mannosyl phosphorylation of N-glycans.

47. The substantially pure culture of claim 39, wherein the  $\alpha$ -1,2-mannosidase is the *Saccharomyces cerevisiae* MNS1 polypeptide or the *S. cerevisiae* MNS1 polypeptide comprising one or more amino substitutions selected from the group consisting of R273L, R273G, R269S, and S272G.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 9,206,408 B2  
APPLICATION NO. : 13/620306  
DATED : December 8, 2015  
INVENTOR(S) : Callewaert et al.

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the claims

Column 125, Line 38 - Claim 9, delete "beta-Dmannosidase," and insert -- beta-D-mannosidase, --, therefor.

Column 125, Line 39-40 - Claim 9, delete "alpha-Nacteylgalactosaminidase," and insert -- alpha-N-acetylgalactosaminidase, --, therefor.

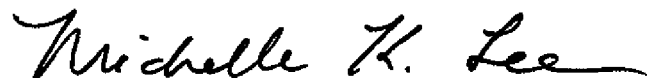
Column 125, Line 42 - Claim 9, delete "beta-D-glucoronidase," and insert -- beta-D-glucuronidase, --, therefor.

Column 125, Line 43 - Claim 9, delete "alphaneuraminidase," and insert -- alpha-neuraminidase, --, therefor.

Column 125, Line 44 - Claim 9, delete "sphingomyelinase," and insert -- sphingomyelinase, --, therefor.

Column 127, Line 5 - Claim 35, delete "the alpha" and insert -- the --, therefor.

Signed and Sealed this  
Twenty-ninth Day of March, 2016



Michelle K. Lee  
*Director of the United States Patent and Trademark Office*